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REMARKS

Reconsideration is requested.

Claims 1-42 have been canceled, without prejudice.

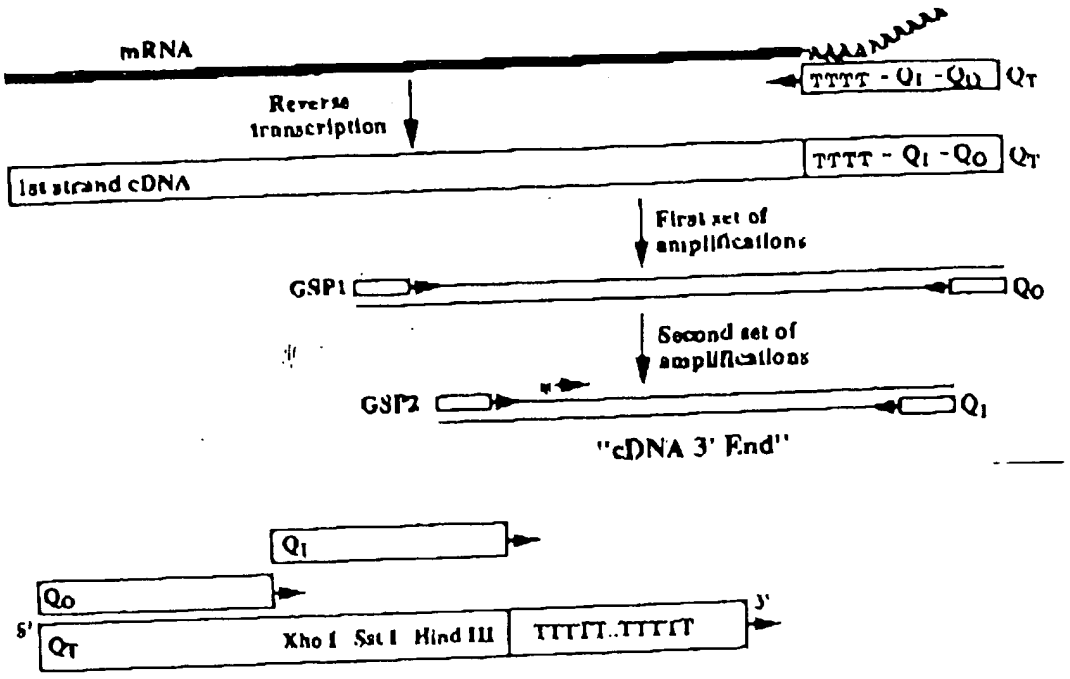
Claims 43-61 have been added and are pending. No new matter has been added.

Page 1 of the specification provides support, for example, for the claimed method of an in vitro diagnostic method of pathologies associated with gene rearrangements. See, page 1, lines 20-22. Leukemias are specifically described in the following paragraph, as well as at page 12, lines 13-14 of the specification. The invention is described, for example, at page 3, line 4-5 as involving detection of rearrangements with a particular partner gene, as recited in claim 43. Moreover, page 3, lines 18-24 of the specification refers to random amplification of fusion genes implicating the target gene, as reflected in claim 43.

The specification describes at page 7, lines 11-14, the use of reverse transcription of RNA extracted from a sample and amplification of cDNA, as recited in claim 43. Page 7, lines 19-22 of the specification provides further support for this aspect of the claimed invention.

The Examiner is urged to appreciate in this regard that steps (a)-(c) of claim 43 generally describe what one of ordinary skill in the art will appreciate as a method of rapid amplification of cDNA ends, or RACE technique, with an "anchored primer". Specifically, the attached excerpt from "PCR PRIMER: A LABORATORY MANUAL" Dieffenbach and Dveksler (Eds.) Cold Spring Harbor Laboratory Press, 1995,

describes, in a section authored by Michael A. Froham (pp. 381-384, copy attached), the following example of this procedure:



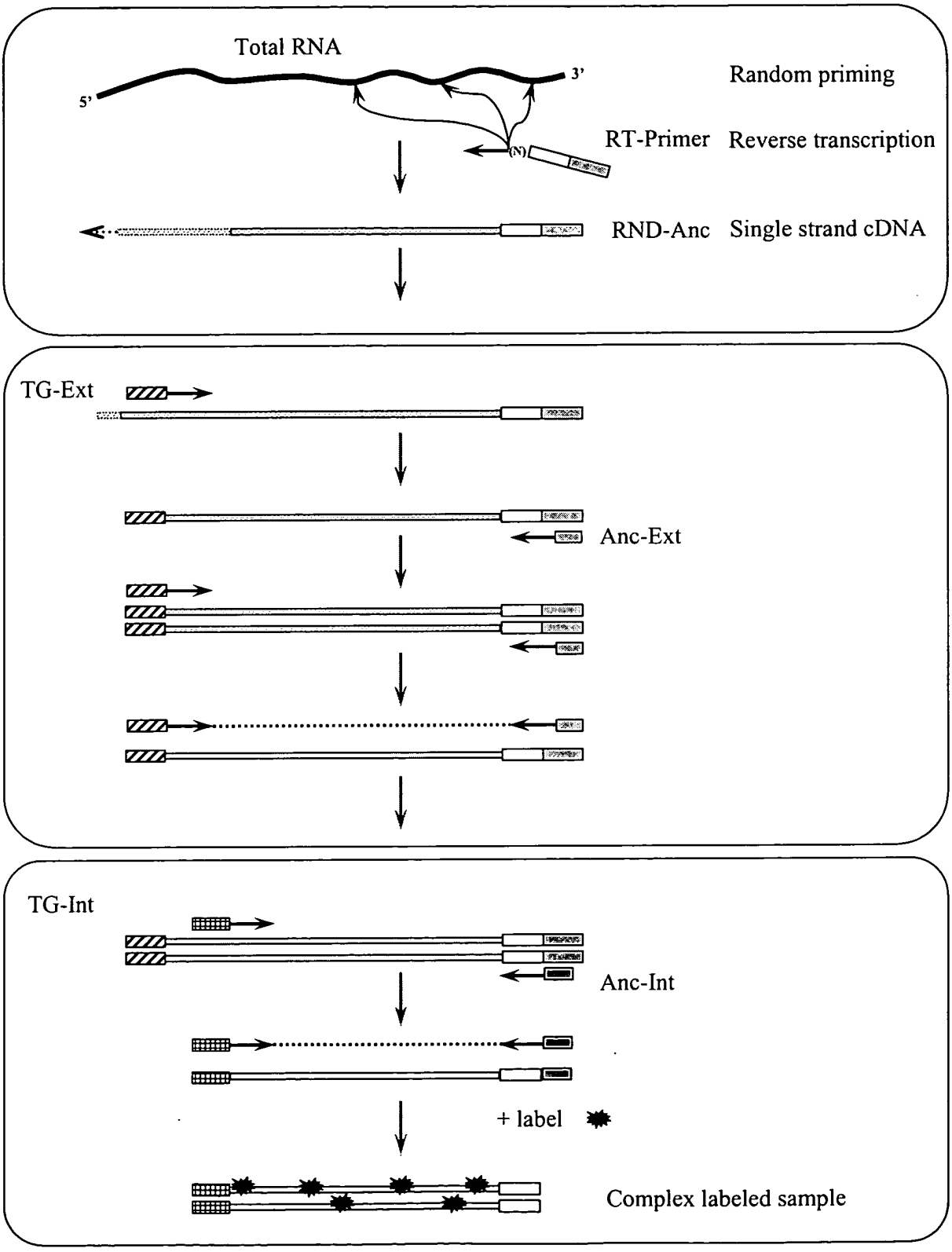
wherein 3' end partial cDNA clones are generated from mRNA which has been reverse transcribed using a "hybrid" primer (Q_T) that, in this example, consists of a 17 nucleotides of oligo (dT) followed by a "unique" 35-base oligonucleotide sequence (Q_1-Q_0) "which in many reports is denoted as an "anchor" primer." Amplification is performed according to this reference using a primer containing a part of this sequence (Q_0) that binds to each cDNA at its 3' end, and using a primer derived from the gene of interest (GSP1). A second set of amplification cycles is described in the reference using "nested" primers "(Q_1 and GSP2) to quench the amplification of nonspecific products.

One of ordinary skill in the art reviewing the present specification, and the references at anchored PCR at page 3, lines 18-24 and page 4, lines 3-11, and elsewhere, of the specification would appreciate as much.

The description of a "single pair of primers" in page 4, line 7 of the specification is a basis for the first primer pair of claim 43, for example. The description of a "specific DNA primer of the gene liable to be involved in a fusion gene" at page 4, lines 8-9 of the specification is believed to support the second primer of claim 43. The description of "a complementary random primer" at page 4, lines 9-10 of the specification, taken in context of the paragraph reference to anchored PCR, will be recognized as a basis for the third primer of claim 43, for example. Further support for the use of anchored primers is found at page 5, line 14 of the specification. Moreover, the 5' alternative described in figure 2b of the attached reference is described in the paragraph spanning lines 14-19 on page 5 of the specification. The inclusion of the nested amplification of claim 43 is described, for example, at page 9, lines 2-4 of the specification. The random primer of the nested amplification, i.e., the fifth primer of claim 43, is described at page 9, lines 4-6 of the specification (i.e., "The random primer is advantageously selected as complementary to the oligonucleotides cassette used on the reverse transcription step."), or similar to Q_1 from the above schematic taken from Figure 2a of Frohman.

The use of detectably labeled nucleotides and detection with probes to known fusion partners is described on page 5, line 22 through page 6, line 23, of the specification.

The invention of claim 43 may be schematically described as follows:


Total RNA
5'
3'
Random priming
RT-Primer
Reverse transcription
RND-Anc
Single strand cDNA
TG-Ext
Anc-Ext
TG-Int
Anc-Int
+ label
Complex labeled sample

wherein TG-Ext is a Target Gene External Primer, TG-Int is a Target Gene Internal Primer, RT-primer is a Reverse transcription random-anchored primer, "RND-Anc" is an anchored primer comprising a random sequence which according to its sequence will hybridise on various locations of the RNA, and a Tail or anchor which is a unique sequence chosen to which the Anc-Ext will anneal at the 1st anchored PCR (PCR I Amplification). Anc-Ext is a primer of known sequence annealing to the single strand cDNA. Anc-Int is a primer of known sequence annealing to the PCR product "internally", i.e.; closer to the center of the cDNA than the Anc-Ext.

Claim 43, as well as all of the pending claims, are submitted to be supported by an adequate written description which teaches one of ordinary skill in the art how to make and use the claimed invention.

The details of claim 44 are described, for example, on page 4, lines 23-26 of the specification.

The details of claims 45 and 51 find support, for example, at page 5, line 28 through page 6, line 3 of the specification.

The details of claim 46 find support, for example, at page 6, lines 4-13 of the specification.

The details of claim 47 and 52, find support, for example, at page 6, lines 4-5 and page 7, line 7 of the specification.

The details of claim 48 find support, for example, at page 7, lines 25-28 of the specification.

The details of claim 49 find support, for example, at page 8, lines 23-25, and Example 3, beginning on page 18 of the specification.

The details of claim 50 find support, for example, in Example 3, beginning on page 18 of the specification.

The details of claim 53 find support, for example, at page 9, lines 16-20 of the specification.

The details of claims 54-56 find support, for example, at page 1, lines 23-29, and page 12, lines 15-21 of the specification.

The details of claims 57-61 find support, for example, in originally-filed claims 14 and 15, as well as the specification at page 1, line 21, page 3, lines 18-24, page 4, lines 1-2, page 10, lines 9-10, page 10, last line through page 11, line 24, and Example 3, beginning on page 18 of the specification.

DNA chips and miniaturized supports are described as detection method and kit supports throughout the specification for numerous gene rearrangements. See, for example, page 7, lines 3-7 of the specification. Attached is a copy of product literature for the applicant's assignee, IPSOGEN, describing the commercially available product based on the presently claimed method. A sample of this chip was shown to the Examiner during the interview with the Examiners. A chip embodiment is specifically recited in claims 47, 52 and 60.

The Section 112, first paragraph, rejection of claims 17-21, 24-27, 29-32, 36-39 and 40-42, is moot in view of the above. The pending claims are submitted to be supported by an adequate written description, for many of the reasons detailed above.

The indiscriminate amplification reverse transcribing of the claims is supported by the specification, as will be appreciated by one of ordinary skill in the art. Contrary to the Examiner's assertions on the bottom of page 2 of Paper No. 25, the specification specifically describes at page 3, line 20, indiscriminate amplification of all types of fusion genes implicating the target gene. As noted above and in the attached, one of ordinary skill in the art well appreciated at the time of the present invention the method of anchored PCR for amplification of cDNA. Moreover, the specification describes, for example, at page 3, lines 19-20, the use of a single pair of primers for the anchored PCR to perform the indiscriminate amplification.

Claim 48, for example, has been drafted with the Examiner's comments in the 1st and 2nd full paragraphs of page 3 of Paper No. 25 in mind. Claim 48 is believed to be adequately described in the specification.

As for the Examiner's comment in the 3rd full paragraph of page 3 of Paper No. 25, the Examiner is requested to see the above discussion as well as the attached, relating to the indiscriminate amplification aspect of the claimed invention.

The specification has been amended to include the requested title "Detailed Description of the Invention. See, page 3 of Paper No. 25.

The Examiner's comment in ¶ B) of Page 4 of Paper No. 25 has been taken in to account when drafting the new claims.

The Section 112, second paragraph, rejection of claims 17-21, 24-27, 29-39 and 40-42, is moot in view of the above. The pending claims are submitted to be definite. Consideration of the following in this regard is requested.

The Examiner's comment in ¶ 7A) of Page 4 of Paper No. 25 has been taken in to account when drafting the new claims.

The Examiner's comment in ¶ 7B) of Page 4 of Paper No. 25 is noted and the Examiner is requested to see the above and attached in this regard.

The Examiner's comment in ¶ 7C) of Page 4 of Paper No. 25 has been taken in to account when drafting the new claims. The claim recitations are submitted to find proper antecedent basis.

The Examiner's comment in ¶ 7D) of Page 5 of Paper No. 25 has been taken in to account when drafting the new claims. The claims distinguish the separate primers by name and function in a manner which will be distinguishable by one of ordinary skill in the art. In response to the Examiner's query, the applicant notes that the random repeated nucleotide primer of an embodiment of claim 48 is totally random.

The Examiner's comment in ¶ 7E) of Page 5 of Paper No. 25 has been taken in to account when drafting the new claims. The claims are submitted to positively recite active method steps, as apparently required by the Examiner.

The Examiner's comment in ¶ 7F) of Page 5 of Paper No. 25 has been taken in to account when drafting the new claims. As noted in the attached and as explained above, the use of nested amplification primers in second stage amplification was well known to one of ordinary skill in the art at the time the present application was filed. The relationship between the various primers in such a method was also well know and appreciated. The claims are submitted to be definite.

The Examiner's comment in ¶ 7G) of Page 5 of Paper No. 25 has been taken in to account when drafting the new claims. The pending claims do not make reference to a complex, as objected to by the Examiner.

The Examiner's comment in ¶ 7H) of Page 6 of Paper No. 25 has been taken in to account when drafting the new claims. The claim recitations are submitted to find proper antecedent basis.

The claims are submitted to be definite.

The Section 102 rejection of claims 17-18, 20 and 39 over Ratech et al (Am J of Clin Path. 1993, 100:527-533) is moot in view of the above. The claims are submitted to be patentable over Ratech and consideration of the following in this regard is requested.

Initially, the applicants note that Ratech teaches a method of 5' anchored PCR (see, for example, figure 1, page 528 of the reference) which is preceded by a dA-tailing step, whereas the presently claimed invention is directed to a method of 3' anchored PCR. More importantly, Ratech teaches the use of cloning and sequencing steps to identify the PCR2 products (see, for example, page 530, col. 2, ¶2). Ratech does not describe therefore a method in which a partner gene is identified by hybridizing the PCR products, i.e., without requiring cloning and sequencing.

Moreover, even at this stage, Ratech has not identified the PCR2 products but rather requires a 3rd PCR (i.e., PCR3) followed by Southern hybridization wherein PCR3 products are migrated on a gel and a unique band is generated, transferred and hybridized with a specific internal probe CDR2 which is radioactive, to enhance

sensitivity. See, page 530, right column, last sentence and Figure 2. In the presently claimed invention, the PCR products are labeled during amplification and then hybridized to detect rearrangements of the target gene.

Ratech fails to teach or suggest the claimed invention.

Moreover, Ratech is not able to selectively detect rearrangements of the target gene. Rather, in order to determine whether a rearrangement has occurred, Ratech requires cloning and sequencing each of the PCR2 products.

The claims are submitted to be patentable over Ratech.

The Section 102 rejection of claims 32 and 37 over Morris (U.S. Patent No. 5,770,421) is moot in view of the above. The claims are submitted to be patentable over Morris. Consideration of the following in this regard is requested.

The Examiner's assertion that the specification does not define an "anchored primer" (see, page 8, Paper No. 25), even if true, which it is not, is not required as the term was well known to those of ordinary skill in the art and recognized to define a specific species of primers. The Examiner's reliance on seemingly general primers as providing the primers of the claimed kits is, with due respect, submitted to be inappropriate. The Examiner will appreciate that Morris teaches, at best, two specific primers, one for NPM and the other for ALK, neither of which are an anchored primer according to the presently claimed invention. Moreover, the probes identified by the Examiner in column 20, lines 42-49 of Morris are themselves labeled whereas in the presently claimed invention the probes are not required to be labeled.

The claims are patentable over Morris.

The Section 103 rejection of claim 19 over Ratech in view of Fodor (U.S. Patent No. 6,309,822) is moot in view of the above amendments. The claims are submitted to be patentable over the cited combination of art. Consideration of the following in this regard is requested.

The deficiencies of Ratech are noted above.

Even if one of ordinary skill in the art were to combine the teachings of Ratech with Fodor, it is unclear from the art where Fodor's array would be used in the Ratech process. The Examiner indicates that it would have allegedly been obvious to have "included the step of detecting fusion partners using the benefits stated by Fodor of providing a more efficient and sensitive detection assay." See, page 10 of Paper No. 25. The Examiner has not indicated what aspect of Ratech's process would be supplemented or eliminated by the array of Fodor, and why. For example, Ratech teaches each of cloning, sequencing and a third polymerase chain reaction amplification (i.e., PCR3), which are not required by the presently claimed invention. There is no suggestion and/or motivation in either Ratech or Fodor to modify Ratech at any step to include an array of Fodor. The Examiner's combination of art is, with due respect, submitted to have been combined with an impermissible use of hindsight. The combination of the cited art is, at best, an invitation to further experiment and fails to establish a *prima facie* case of obviousness. Moreover, as noted above, Ratech teaches a 5' anchored primer reaction which does not suggest the presently claimed invention.

The claims are submitted to be patentable over the combination of Ratech and Fodor.

The Section 103 rejection of claims 24-26 and 29 over Ratech in view of Felix (U.S. Patent No. 6,368,791) is moot. The claims are submitted to be patentable over the cited combination of art. Consideration of the following in this regard is requested.

The deficiencies of Ratech have been noted above. While Felix may mention MLL translocations, the applicant believes that Felix describes, at best, a method for panhandle PCR amplification requiring digestion, ligation, cloning and sequencing steps. Felix therefore suffers from some of the same deficiencies of Ratech. Even if motivation existed in the art to combine Ratech and Felix, the combined art would not have made the presently claimed invention obvious. The claims are submitted to be patentable over the combination of Ratech and Felix.

The Section 103 rejection of claims 27 and 40-42 over Ratech in view of Felix (U.S. Patent No. 6,368,791) and Hoeltke (Cellular and Molecular Biology (1995) 41(7) 883-905) is moot The claims are patentable over the combination of cited art and consideration of the following in this regard is requested.

The deficiencies of Ratech and Felix have been noted above. Hoeltke fails to cure these deficiencies. The Examiner has apparently applied Hoeltke merely because of an alleged mention of digoxygenine in the context of PCR. The Examiner has not indicated however how the cited combination of art would have motivated one of ordinary skill in the art to make the presently claimed invention. As noted above, Ratech requires the use of cloning, sequencing and further amplification, at a minimum,

which are not required by the presently claimed invention. The Examiner's secondary references fails to cure these, and many other, deficiencies of Ratech. The claims are submitted to be patentable over Ratech, Felix and Holtke.

The Section 103 rejection of claims 30-31 over Ratech in view of Denny (Cancer Investigation (1996) 14(1) 83-88) is moot in view of the above. The claims are submitted to be patentable over the cited art. Consideration of the following in this regard is requested.

The deficiencies of Ratech are noted above.

The teachings of Denny fail to cure these deficiencies. The applicant submits that Denny teaches, at best, a marginal reference to using PCR for rearrangement detection. Denny further teaches the importance of detecting rearrangements in genes associate with Ewig's sarcoma. There is no suggestion however in Denny or Ratech to combine the reference to produce the presently claimed invention. There is no suggestion, for example, in Denny to alter the Ratech method which, as noted above, involved cloning, sequencing and further amplification, at a minimum, which are not required by the presently claimed invention. The claims are submitted to be patentable over Ratech and Denny.

The Section 103 rejection of claims 36 and 38 over Morris (U.S. Patent No. 5,770,421) in view of Smith (U.S. Patent No. 5,753,439) is moot in view of the above amendments. The claims are submitted to be patentable over the cited art. Consideration of the following in this regard is requested.

The deficiencies of Morris have been described above. Smith is understood to teach a method of determining the sequence and/or length of a target sequence, in particular repeated sequences. It is not clear where Smith teaches however the detection of fusion genes, such that motivation to combine the reference, absent an impermissible use of hindsight, appears to be lacking. Moreover, as noted above, one of ordinary skill in the art would appreciate that neither of Morris' specific primers are an anchored primer, as required by the presently claimed invention.

The claims are submitted to be patentable over the combined teachings of Morris and Smith.

The Section 103 rejections of claims 32 and 37 over the combination of either Rassenti (Annals of the NY Academy of Science (1995) 764: 463-473) and Stratagene Catalog (1988), or Ratech, Fodor and the Stratagene Catalog, are moot in view of the above. The claims are submitted to be patentable over the cited art. Consideration of the following in this regard are requested.

Both of these rejections again are based on the Examiner's continued misinterpretation of the level of skill in the art with regard to an appreciation of the definition and operation of an anchored primer and anchored PCR at the time the present invention was made. Ratech, as repeatedly indicated above, teaches a 5' anchored PCR and further laborious and complicated steps of cloning, sequencing and further amplification which are contrary to the elegance and simplicity of the presently claimed invention. The arrays of Fodor and reagents of the Stratagene catalog would

not have cured these deficiencies of Ratech, and/or led one of ordinary skill in the art to make the presently claimed invention from Ratech.

Moreover, Rassenti is believed to disclose a method for analysis of Ig repertoires by detection of rearrangements, as opposed to "fusion genes" as suggested by the Examiner at page 14 of Paper No. 25. The reference is further believed to teach the use of two anchored primers (see, page 465, 1st ¶). The Stratagene Catalog is not believed to cure any of the deficiencies of the primary references.

The claims are submitted to be patentable over the cited art.

The claims are submitted to be in condition for allowance and a Notice to that effect is requested. The Examiner is requested to contact the undersigned in the event anything further is required.

Respectfully submitted,

NIXON & VANDERHYE P.C.

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For Life Science Research Only – Not Intended for Diagnostic Use.

MLL Fusion*Chip*™ Kit

The *MLL* Fusion*Chip*™ is a molecular diagnostic device designed to determine whether a translocation is present on the MLL gene, to identify accurately the MLL partner gene and relative breakpoint in patients with acute leukaemia.

Ref: MLFC-01
Five Tests

Store at:
Vial T1, T2, T3, T4 and B1: **-20°C**
MLL **Fusion*Chip*™**, Vial B2 and B3: **Room Temperature**
Vial B4, B5 and T5: **+ 4°C**
Caution: Reagent B5 is photosensitive and must be stored in the dark

Instructions for Use
Version 02, July 2003.

Ipsogen
CancerProfiler

Ipso gen
CancerProfiler

MLL Fusion*Chip*™
Instructions for Use
First Edition

MLL Fusion*Chip*™
For MLL fusion transcript detection
The kit contains sufficient components to perform five tests

Ref: MLFC-01

Table of contents:

1. Intended Use

The *MLL* Fusion*Chip*™ is a molecular analytical tool designed to determine whether a translocation is present on the MLL gene, to identify accurately the MLL partner gene and the relative breakpoint on the partner gene.

Disclaimer: **For Life Science Research Only – Not Intended for Diagnostic Use.**

2. Background

A variety of chromosomal aberrations have been reported in haematological malignancies. Recent studies have demonstrated that several chromosomal rearrangements and molecular abnormalities are strongly associated with distinct clinical subgroups and are predictive of clinical features and therapeutic responses. The 11q23 translocation is a frequent cytogenetic abnormality found in haematological malignancies, occurring in 5–6% of patients with AML, 7–10% of patients with ALL, 60–70% of infants with acute leukaemia, and in most patients with therapy-related leukaemia induced by inhibitors of topoisomerase II. At least 40 chromosomal regions for partners of 11q23 have been observed. The *MLL* gene (also called *ALL-1* or *HRX*) has been identified in 11q23 translocations, and at least 32 partner genes for *MLL* have been cloned from leukaemia cells with various 11q23 translocations that formed fusion transcripts with *MLL*. The functions of some genes have been clarified, including those for a Ras-binding protein (*AF6*), an RNA polymerase II elongation factor (*ELL/MEN*), transcriptional coactivator/ histone acetyltransferase (*CBP* and *P300*), and *ABL* and eps8-binding protein (*ABI-1*). Recently, it was shown that both the "knock-in" of *AF9* into *MLL* and the *MLL-ENL* fusion caused myeloid malignancies in mice.

3. Technological Principle

The test is based on the amplification and detection of fusion transcripts involving the *MLL* gene by reverse transcription of the patient RNA followed by an anchored PCR (sample processing) and a multiplexed detection on a biochip (sample hybridisation and analysis). This proprietary (Ipsogen patent application # WO99/23251) methodology was developed and optimised to allow the comprehensive analysis of gene rearrangements of prognostic significance in the etiology of leukaemia. The sample-processing step ensures that all rearrangements of a given master gene (*MLL* in this product) can be consistently detected independently of transcript length and position of the breakpoint involved in the fusion transcript. Briefly, random reverse transcription on a 3' primer is performed on total RNA. The resulting cDNA is amplified with an anchored PCR (PCRI) from a *MLL* specific primer on the 5' side (upstream of known translocation points) and from the 3' flanking region with a primer specific of the anchor used for the RT step. The amplified cDNA is subsequently labeled by a biotinylated nucleotide, and the complexed probe hybridised on the *MLL* Fusion*Chip* ™. The biochip is designed to achieve a simple direct visual reading of test results (Ipsogen patent application FR0213487). A colourimetric reaction visually shows the presence of an *MLL* translocation, and, if any, the partner gene involved in the fusion transcript and estimation of the breakpoint position.

4. Technological Specifications

4.1. Spotted oligonucleotides

Long oligonucleotides (50 mers) corresponding to translocated regions of *MLL* and its known potential partners are spotted on the chip. Normal *MLL* transcripts or molecules synthesized from the 5' region of *MLL* are specifically amplified, hybridised with the spotted oligos, and identified on the chip. The biotinylated oligonucleotide included on the chip serves as an internal control for:

- ✓ colourimetric reaction
- ✓ chip orientation
- ✓ accurate test interpretation

Each partner is represented on the chip by a series of oligonucleotides selected to describe the region known to be implicated in gene fusion events. When possible, at least one oligonucleotide representing a region upstream of the described break-point for each partner (first spot of each line) is include to serve as a negative control (see figure 1).

4.2. Partners and Fusion Points

Breakpoints on *MLL* span an 8Kb genomic region, from exon 5 to exon 11. Breakpoints on the partner are highly variable, ranging from regions located near the NH_2-term in *ENL, AF1q or MSF*, as well as in close proximity of the COOH-term on *AF9*. The MLL specific primers, used for PCR-I and PCR-II amplification, represent sequences of the 4c exon of MLL (upstream of the most 5' known breakpoint).

Table 1 : Partners and Translocations

PARTNER	Translocation	PARTNER	Translocation
ABI1 (10p11)	t(10;11)(p11.2;q23)	*ELL* (19p13)	t(11.19)(q23;p13.1)
AF10 (10p12)	t(10;11)(p12;q23)	*ENL* (19p13)	t(11.19)(q23;p13.3)
AF15q14 (15q14)	t(11;15)(q23;q14)	*FBP17* (9q34)	t(9;11)(q34;q23)
AF17 (17q21)	t(11;17)(q23;q21)	*GAS7* (17p13)	t(11;17)(q23;p13)
AF1p (1p32)	t(1;11) (p32;q23)	*GPHN* (14q24)	t(11;14)(q23;q24)
AF1q (1q21)	t(1;11) (q21;q23)	*GMPS* (3q25)	t(3;11)(q25;q23)
AF3p21 (3p21)	t(3;11)(p21;q23)	*GRAF* (5q31)	t(5;11)(q31;q23)
AF4 (4q21)	t(4;11)(q21;q23) Variable breakpoints	*hCDCRel-1* (22q11)	t(11;22)(q23;q11)
AF5q31 (5q31)	ins(5;11)(q31;q13q23)	*LAF4* (2q21)	t(2;11)(q21;q23)
AF6 (6q27)	t(6;11)(q27;q23)	*LARG* (11q23)	Normal karyotype
AF6q21 (6q21)	t(6;11)(q21;q23)	*LCX* (10q22)	t(10;11)(q22;q23)
AF9 (9p22)	t(9;11) (p22;q23) Variable breakpoints on both genes	*LPP* (3q28)	t(3;11)(q28;q23)
AFX1 (Xq13)	t(X;11)(q13;q23)	*MSF* (17q25)	t(11;17)(q23;q25)
CBP (16p13)	t(11;16)(q23;p13)	*P300* (22q13)	t(11;22)(q23;q13)
CDK6 (7q21)	t(11;7)(q23;q21)	*RARA* (17q12)	t(11;17)(q23;q12)
EEN (19p13)	t(11;19)(q23;p13)	*SEPTIN* (Xq22)	t(X;11)(q22;q23)

4.3. Chip Format

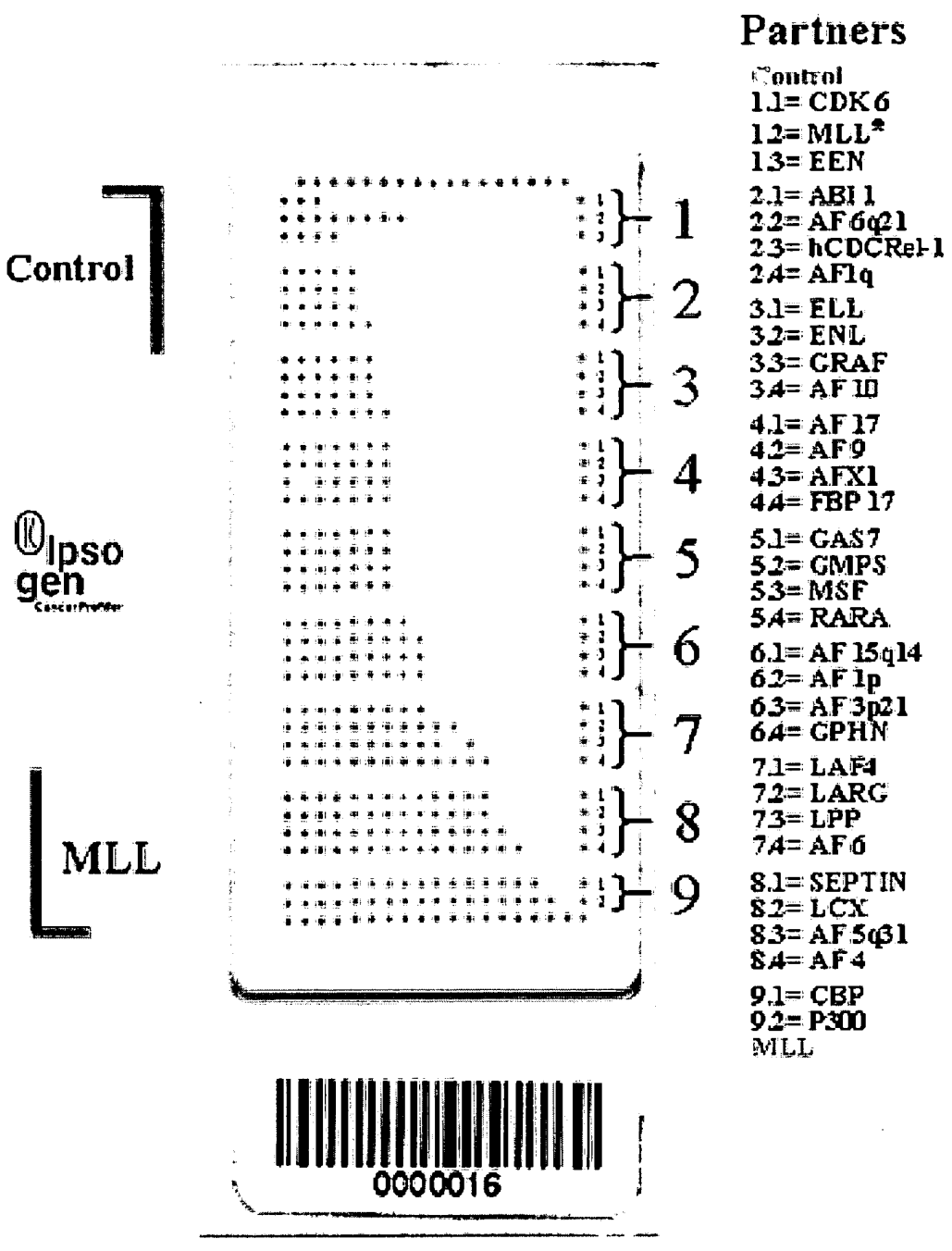


Figure 1

- ✓ Inverted L-shaped biotinylated control.
- ✓ L-shaped master gene oligonucleotide (*MLL*).
- ✓ Horizontal partner's oligonucleotide.

*: If position 1.2 is positive, this may indicate the presence of an *MLL* internal duplication.

5. Reagents & Instruments

5.1. Materials provided

Table 2 : Kit contents: This kit contains sufficient reagents to process 5 samples.
Unopened kit components are stable at the storage temperature below until the expiration date printed on the label.

Vial/ Device	Use in step	Label	Volumes	Use / comments	Storage temperature	Part Number
T1	RT-PCR	RT primer	15 µL	Ready to use primer solution for cDNA synthesis.	-15°C to -25°C	IP-PF-000002
T2	PCR I	PCR I primer mix	25 µL	Ready to use primer solution for the first PCR amplification: PCR I.	-15°C to -25°C	IP-PF-000003
T3	PCR II	PCR II primer mix	30 µL	Ready to use primer solution for labeling PCR step: PCR II.	-15°C to -25°C	IP-PF-000004
B1	Hybridisation	Hybridisation Buffer 1X	60 ml	Ready to use hybridisation buffer. Heat solution to 65°C before use.	-15°C to -25°C	IP-PF-000009
T4	Hybridisation	ssDNA	600 µl	Denature immediately prior to use.	-15°C to -25°C	IP-PF-000011
MLL-FC	Hybridisation	*MLL* Fusion *Chip*™	5 chips	Store in clean and dry atmosphere. Handle with gloves.	Room Temperature	IP-PF-000001
B2	Detection	Washing Buffer 1	2X100 ml	Ready to use. Check for SDS precipitation.	Room Temperature	IP-PF-000010
B3	Detection	Washing Buffer 2 (10X)	50 ml	10 X Stock solution. Dilute to 1X immediately prior to use in ddH2O.	Room Temperature	IP-PF-000012
B4	Detection	Blocking Buffer (5X)	40 ml	5 X Stock solution. Dilute to 1X immediately prior to use in ddH_2O.	+ 4°C	IP-PF-000013
T5	Detection	Conjugate	20 µl	Ready to use. Stable at 2-8°C. Store protected from light.	+ 4°C	IP-PF-000014
B5	Detection	Substrate	20 ml	Ready to use. Stable at 2°- 8°C. Store protected from light.	+ 4°C	IP-PF-000015

5.1.1. Handling and storage

See above storage conditions. Expiration dates for each of the unopened components are indicated on the individual component labels. These storage conditions apply to both opened and un-opened components. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.
Store all kit components in original containers.
NB: There are three different temperature storage sections within the kit (room temperature, 4°C and -15°C to -25°C). Please store each part of the kit at the correct temperature according to the labelling on each section.

5.1.2. Warning

- ✓ Buffer B2 and B4 contains 0.09% sodium azide.
- ✓ Buffer B1 and B2 contains SDS.
  - R 21/22: Harmful in contact with skin and if swallowed: -R 36/38: Irritating to the eyes and skin
- ✓ Buffer B5: Avoid contact and inhalation. Target organ(s): Central nervous system, Kidneys.
  - R61: May cause harm to the unborn child - R 20/21: Harmful by inhalation and in contact with the skin
  - R 36: Irritating to the eyes and skin
- ✓ The hybridisation chamber may be reused numerous times (about ten times), but not indefinitely, as the plastic may slightly deform after repeated high-temperature exposure. Please, refer to specific directions for use.

5.2. Materials required but not provided

5.2.1. Reagents

Table 3 : Validated Additional Reagents

Reagent	Validated reagent (Brand name)	Validated reagent (Provider, reference in Europe)
Reverse transcriptase	Expand™ Reverse Transcriptase [1)]	Roche # 1 785 826
DTT 100 mM	DTT (supplied with Roche RT)	Roche # 1788558
RNase Inhibitor (40U/µl)	RnaseOut™. [2)]	Invitrogen # 10777-019
Taq Polymerase long range	Expand™ Long Template PCR System.	Roche # 1 681 834
100mM dNTP	Set of dNTP, PCR Grade.	Any
Biotin-16-dUTP (1mM)	Biotin-16-2'-deoxy-uridine-5'-triphosphate.	Roche # 1 093 070
PCR grade H_2O, RNase, DNase free	Any	Any
100% Acetic Acid (Analytical reagent)	Any	Any
RT and PCR reaction buffers	5X Expand™ reverse transcriptase buffer and 10X Expand™ long template PCR buffer 3.	Supplied with Roche RT and PCR kits above
Hybridisation chamber	Hybridisation Chamber	IPSOGEN FCHC-01

5.2.2. Equipment

To perform the assay, you will need the following equipment:

- ✓ 0.5ml or 0.2ml PCR tubes RNase- and DNase free
- ✓ Nuclease free aerosol-resistant sterile PCR pipette tips with hydrophobic filters
- ✓ Sterile reaction cups (eppendorf) for preparing dilutions
- ✓ Microcentrifuge equipped for 0.2 ml/0.5ml tubes. Max speed: 13 000 / 14 000 rpm
- ✓ Microliter pipettor dedicated for PCR (1-10µl; 10-100µl; 100-1000µl)
- ✓ Thermal Cycler with heated lid
- ✓ Electrophoresis chamber, agarose
- ✓ Molecular biology grade water for buffer dilution (B3 and B4)
- ✓ DNA molecular weight markers
- ✓ Hybridisation oven (65°C) with orbital agitation
- ✓ Water bath (37°C)
- ✓ Incubator (37°C)
- ✓ Forceps
- ✓ Ice

5.3. Warning and precautions

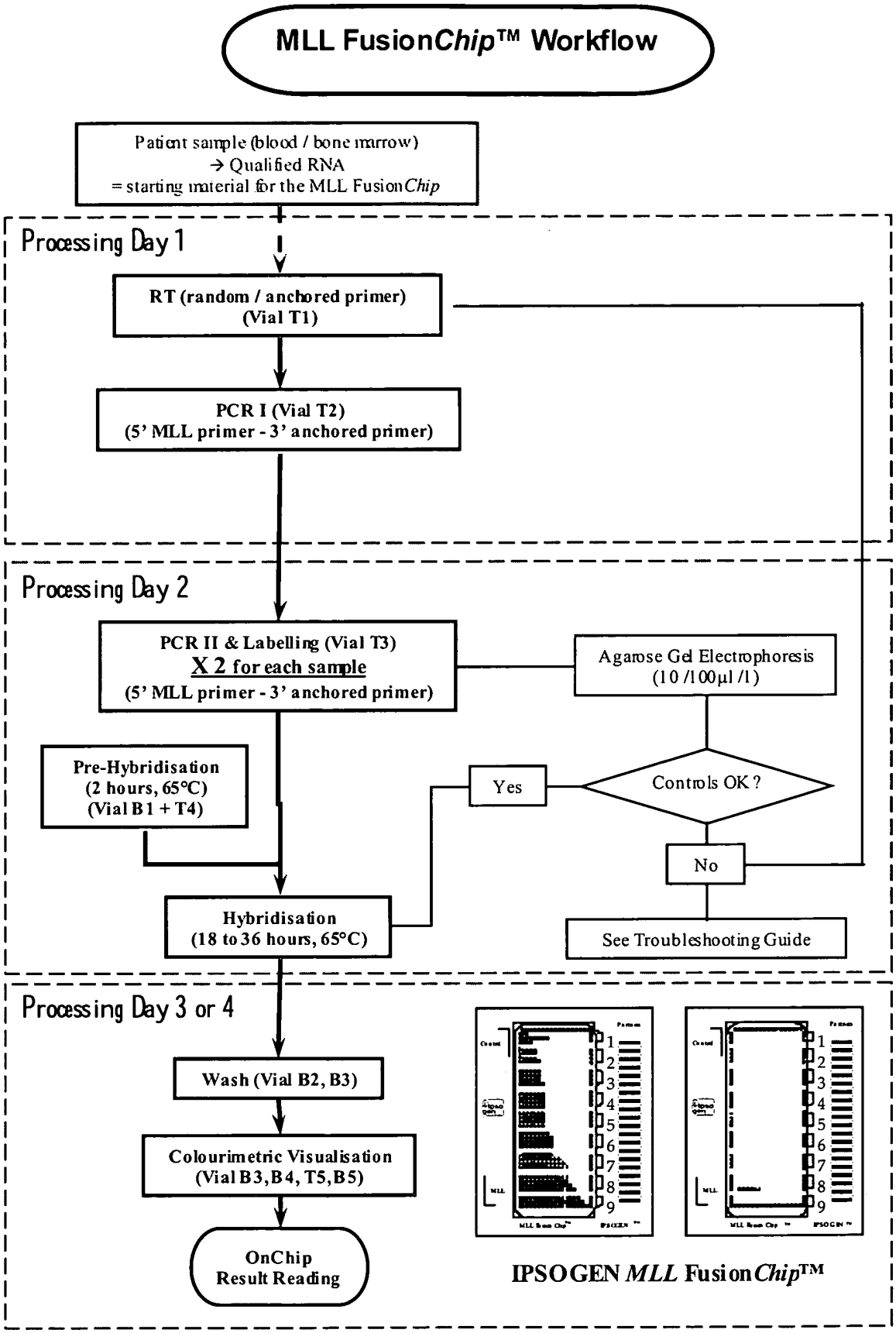
NOTE: The reagents and instructions supplied in this kit have been validated for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may give erroneous or discordant results. Differences in sample processing and technical procedures in the user's laboratory may invalidate the assay results.
The entire assay procedure must be performed under RNAse free conditions.

- Use extreme caution to prevent:
  - RNAse/DNAse contamination, that might cause degradation of the template mRNA and the generated cDNA
  - mRNA or PCR carry-over contamination resulting in false positive signal
- If needed, prepare appropriate aliquots of the kit solutions or additional reagents and keep them separate from other reagents in the laboratory.
- Use nuclease-free labware (e.g. pipettes, pipettes tips, reactions vials) and wear gloves when performing the assay.
- Use fresh aerosol-resistant pipette tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Minimize microbial contamination of reagents to avoid non-specific staining.
- Incubations times, temperatures, or methods other then those specified may give erroneous results.
- Reagents have been optimally diluted. Further dilutions may result in loss of staining or erroneous results.
- The visualisation reagent may be adversely affected if exposed to light. Do not store system components or perform staining in strong light, such as direct sunlight.
- Wear appropriate personal protective equipment to avoid contact with eyes and skin. Refer to the Materials Safety Data Sheet (MSDS) for additional information.
- Human tissues must be handled as if capable of transmitting infections and disposed of with proper precautions, and in compliance with OSHA and/or CAP (or EU equivalent) guidelines.
- Never pipette kit reagents by mouth and avoid contact with skin and mucous membranes. If reagents are exposed to sensitive areas, wash thoroughly with copious amount of water and contact a physician.
- All reagents are formulated specifically for use with this test. No substitutions should be made for optimal performance of this test.
- This product contains sodium azide (NaN_3), a chemical highly toxic in pure form, and may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Refer to state/local laws and pertinent regulatory agencies for appropriate disposal guidelines.

6. Instructions for Use

Before you begin
The user should read these instructions carefully and become familiar with all components prior to use.
The quality of the assay is largely dependent on the quality of input RNA. We therefore recommend analysing the purified RNA by agarose gel electrophoresis or Agilent bioanalyser prior to analysis.

6.1. Procedure



Ex: MLL-AF4 Positive Test

6.2. Protocol overview

Sample material:
Purified total RNA (or messenger RNA (mRNA)) from clinical sample material such as with blood cells or bone marrow.

Table 4: Assay time

Procedure	Time
Step 01: Set up for cDNA synthesis	Approx. **15 min**
Step 02: cDNA synthesis	**90 min**
Step 03: Set up of PCR I	Approx. **15 min.**
Step 04: PCR I amplification	**240 min**. Variable from one thermal cycler to another.
Step 05: Set up of PCR II	Approx. **15 min.**
Step 06: PCR II amplification	**240 min**. Variable from one thermal cycler to another.
Step 07: Agarose gel electrophoresis set up and running	**90 min.**
Step 08: Pre-hybridisation + set up	**150 min.**
Step 09: Hybridisation + set up	**18 to 36 hours.**
Step 10: Washing I	**60 min.**
Step 11: Pre-blocking	**65 min.**
Step 12: Conjugate incubation	**65 min.**
Step 13: Washing II	**45 min.**
Step 14: Detection	**5 to 15 min.**
Step 15: Fixation	**10 min.**

7. Sample treatment protocol

7.1. Probe synthesis

7.1.1. Reverse Transcription

- ❑ Thaw all necessary components and place them on ice.
- ❑ Prepare the following sample mix in a PCR tube on ice:
  Use a sterile RNase- and DNase free tube on ice, preferably a 0.2ml thin-walled PCR tube.

Sample mix	Volume	Final Conc.
Sample RNA to be tested (1 µg)	1 to 9.5 µl	50 ng/µl
Primer **Vial T1** (10 µM)	1.0 µl	0.5 µM
PCR grade nuclease free water	Adjust vol. to 10.5 µl	

- ❑ Mix gently and spin briefly in a microcentrifuge (~10 sec, 10,000rpm, to collect the liquid in the bottom of the tube).
- ❑ Incubate **at 65°C** for **10 min** in a water bath or a thermal cycler.
- ❑ Rapidly cool sample mix on ice for **5 min.**
- ❑ Briefly spin in a microcentrifuge (~10 sec, 10,000rpm, to collect the liquid in the bottom of the tube) and keep the tube on ice.
- ❑ Prepare the following RT pre-mix according to the number of samples being processed.

RT Premix	Vol. for 1 sample	Final Conc.	Premix for 1 sample + 1 control	Premix for 5 samples + 1 control
5X Expand™ reverse transcriptase buffer (first-strand)	4.0 µl	1X	10 µl	26 µl
dNTP (10mM each, to be prepared previously and stored at -20°C in aliquots)	2.0 µl	1 mM	5 µl	13 µl
DTT (100 mM) (supplied with Roche Expand RT)	2.0 µl	10 mM	5 µl	13 µl
RNAse Inhibitor (40U/µl)	0.5 µl	1 U/µl	1.25 µl	3.25 µl
Roche Expand™ Reverse Transcriptase	1.0 µl	2.5 U/µl	2.5 µl	6.5 µl

- ❑ Keep on ice.
- ❑ Prepare the following mix.

Component	Volume
RT Premix	9.5 µl
Sample mix	10.5 µl

- ❑ Mix well and spin briefly (~10sec, 10,000rpm, to collect the liquid in the bottom of the tube).
- ❑ Incubate **at 30°C** on a thermal cycler for **10 min,**
  then immediately for 45 min at 42°C.
- ❑ Cool on ice (to stop the reaction) for 5 min.
- ❑ Briefly spin (~10sec, 10,000rpm, to collect the liquid in the bottom of the tube) the obtained cDNA (keep on ice).
- ❑ Process the following step immediately.

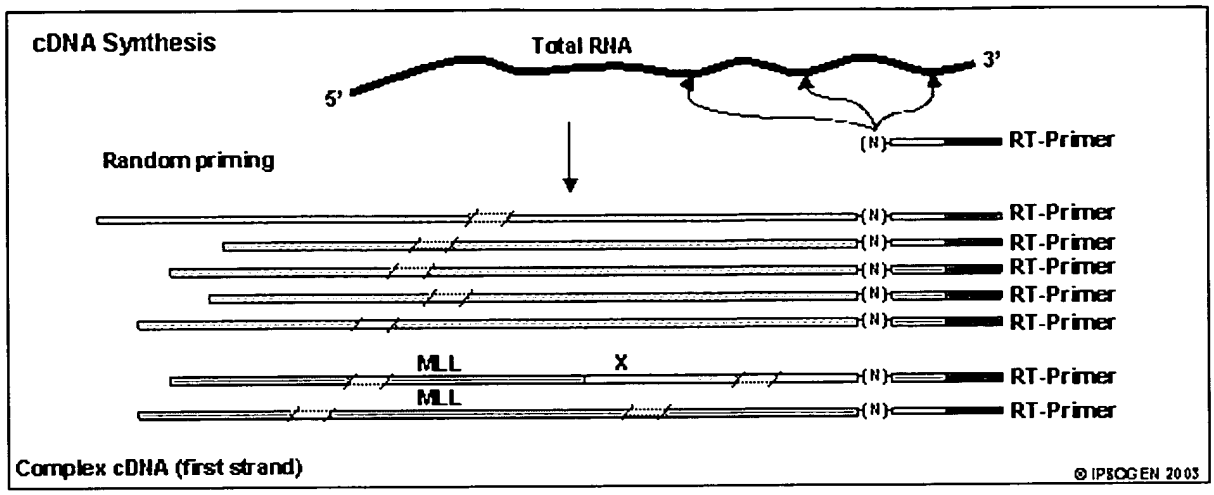


Figure 2

7.1.2. PCR I

- ❑ Thaw all necessary components and place them on ice.
- ❑ Prepare the following **PCRI premix** according to the number of samples being processed.
  Use a sterile RNase- and DNase free tube, preferably a 0.2ml thin-walled PCR tube.
  Warm 10x Expand™ Long Template PCR buffer 3 to room temperature, then mix well.

PCR I premix	Vol. for 1 sample	Final Conc.	Premix for 1 sample + 1 control	Premix for 5 samples + 1 control
Roche 10X Expand™ Long Template PCR Buffer 3	5.0 µl	1 X	12.5 µl	32.5 µl
dNTP (10 mM each, to be prepared previously and stored at -20°C in aliquots)	2.5 µl	500 µM each	6.25 µl	16.25 µl
PCR I primers mix. **Vial T2** (25µM each)	1.0 µl	0.5 µM	2.5 µl	6.5 µl
Roche Expand™ Long Template Enzyme mixture	1.0 µl	0.07 U/µl	2.5 µl	6.5 µl
Adjust vol. to 45 µl with nuclease-free H_2O	35.5 µl		88.75 µl	230.75 µl

To be performed on ice:

- ❑ Label the PCR tubes.
- ❑ Dispense **45 µl of the PCRI** pre-mix per tube.
- ❑ Homogenize PCR I product.
- ❑ Add **5 µl** of the **RT product (cDNA)** obtained in step 7.1.1 above **in the corresponding tube (total volume 50µl).**
- ❑ Mix gently, do not vortex.
- ❑ Place the tubes in the thermal cycler.
- ❑ Run the following program:

PCR I and II program:		
Temperature	**Time**	**Cycles**
Lid 110°C		
94°C	4 min	**X1**
94°C	30 sec	**X10**
60°C	30 sec	
68°C	4 min	
94 °C	30 sec	**X20**
60°C	30 sec	
68°C	4 min + 20 sec increment for each cycle (e.g. cycle n°12 has in addition 20 s; cycle n°13 has in addition 40s (20+20); ...).	
68°C	7 min	**X1**

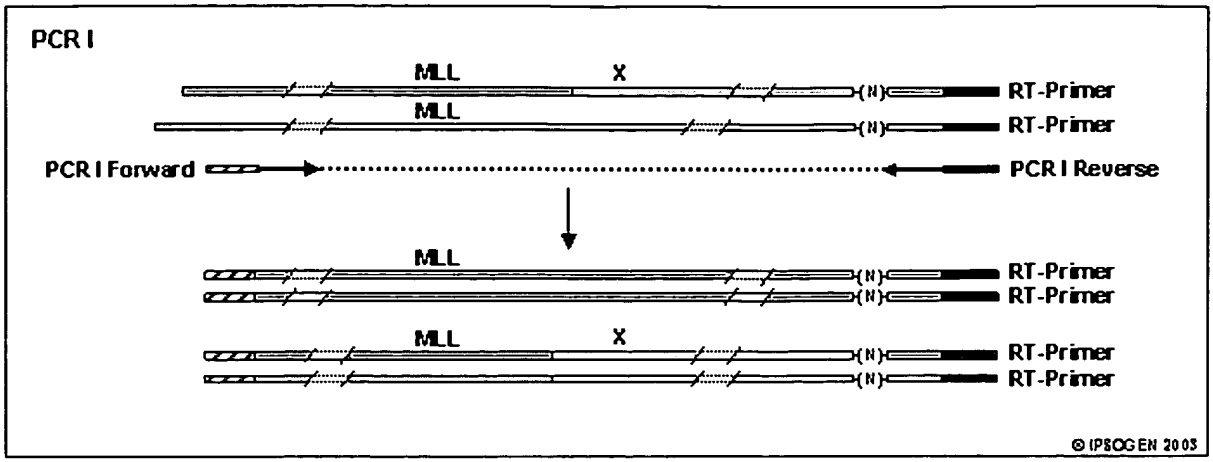


Figure 3

7.1.3. PCRII / labelling

- ❑ <u>For each sample, systematically prepare two PCR-II probes</u> in two separate tubes.
- ❑ Thaw all necessary components and place them on ice.
- ❑ Prepare the following **PCRII pre-mix** according to the number of samples being processed.
  Use a sterile RNase and DNase free tube, preferably a 0.2ml thin-walled PCR tube.
  Warm the 10x Expand™ Long Template PCR buffer 3 to room temperature, then mix well.

PCR II premix	Vol. for 1 reaction	Final Conc.	Premix for 1 sample + 1 control	Premix for 5 samples + 1 control
Roche 10X Expand™ Long Template PCR Buffer 3	5.0 µl	1X	17.5 µl	57.5 µl
dAGC mix (10 mM each, to be prepared previously and stored at -20°C in aliquots)	2.5 µl	500 µM	8.75 µl	28.75 µl
dTTP (10 mM, to be prepared previously and stored at -20°C in aliquots)	2.25 µl	450 µM	7.90 µl	25.90 µl
Roche Biotin-16-dUTP (1mM)	2.5 µl	50 µM	8.75 µl	28.75 µl
PCR II primers mix. **Vial T3** (25µM each)	1.0 µl	0.5 µM.	3.5 µl	11.5 µl
Roche Expand™ Long Template Enzyme mixture	1.0 µl	0.07 U/µl	3.5 µl	11.5 µl
Adjust vol. to 49 µl with H_2O	34.75 µl		121.6 µl	399.6 µl

To be performed on ice:

- ❑ Label a PCR tube for each sample being processed. (<u>**2 / sample**</u>)
- ❑ Dispense **49 µl** per tube **of the PCRII pre-mix.**
- ❑ Add **1 µl of the previously prepared PCRI product (gently mixed before) in the corresponding tubes** (total volume 50µl).
- ❑ Mix gently, do not vortex.
- ❑ Place the tubes in the thermal cycler.
- ❑ Run the same program as for the PCR I. See step 7.1.2

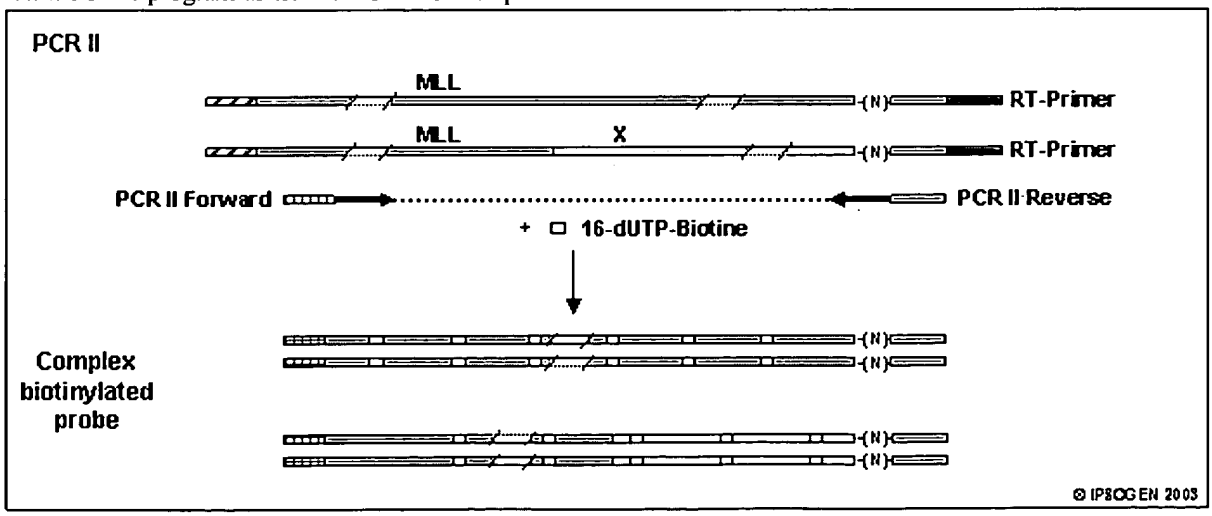


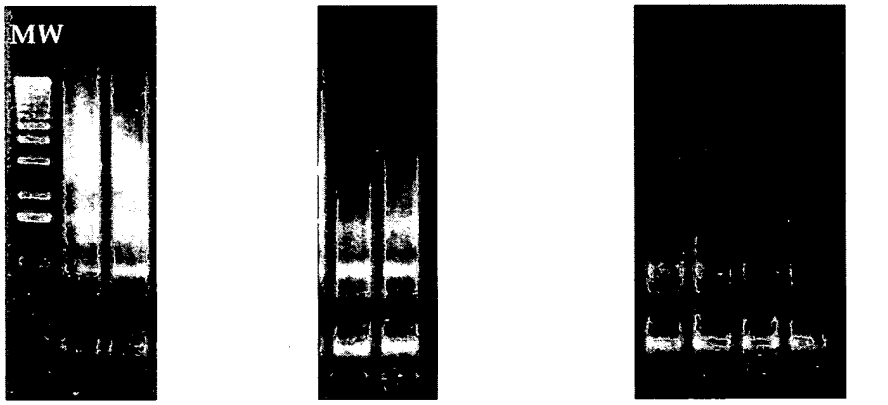
Figure 4

PCR II Quality Control (to be done systematically)
PCR II products (10µl/100µl of the mixed probes) analyzed on a 1% Agarose/ethidium bromide (1µg/ml) gel should show a smear with some discrete bands (variable from one sample to another) ranging from 10-12 Kb to the migration front (see example below, figure 5A). In absence of a smear, **DO NOT PROCESS FURTHER: the sample treatment failed and no interpretable result will be obtained from this test.**
Check for RNA integrity.

Enzymes quality control: We recommend performing a quality control of your enzymes for each batch you receive and prior to use in a *MLL* Fusion*Chip*™ test.

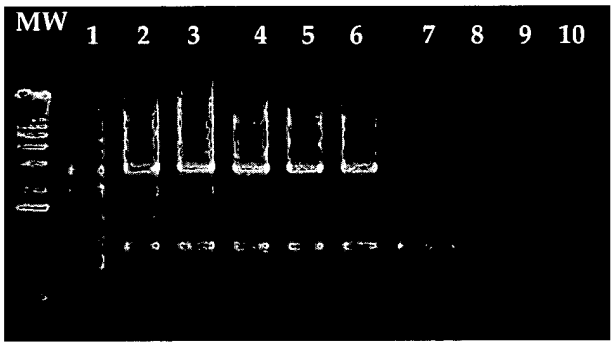
a) Perform a RT reaction (RT-primer, vial T1-Chap 7.1.1) on a validated Control RNA (PBL or cell line, not provided),
b) Then a first "PCR-I" using the T2 primer mix as describe above (chap 7.1.2).
c) This subsequent PCR product (1µl) is then further amplified with a second "PCR-I" (chap 7.1.2) reaction using T3 primer mix. CAUTION: In this case, add 39.5µl H_2O instead of 35.5 in your PCR-I pre-mix.

Note: The kit contains sufficient reagents to perform this additional test.



Expected result A | Poor RT quality B | Poor Taq Polymerase quality C

Figure 5
(PCR II product visualisation on agarose gel)



1, 2, 3, 4, 5 and 6: Probes prepared with good quality and validated control RNA
7, 8, 9, and 10: Probes prepared with poor quality RNA samples.

Figure 6 (PCR II product visualisation on agarose gel)

Cross contamination: Cross contamination most often results in extra bands (specific PCR) or smearing (starting from pits to the migration front (figure 7). It is important to include a negative control (replace the DNA template with PCR-grade H2O but still include the primers) in every PCR experiment to determine if the reagents, pipettes or PCR reaction tubes are contaminated. If possible, set up the reaction and perform the post-PCR analysis in separate areas with separate sets of pipettes.

Figure 7
(PCR II product visualisation on agarose gel)

Example of a cross contamination result

8. *MLL* Fusion*Chip*™

8.1. Hybridisation

8.1.1. Pre-hybridisation

- ❑ Prewarm hybridisation oven to 65°C.
- ❑ Preheat hybridisation buffer (**Buffer B1**) to 65°C.
- ❑ Place the *MLL* Fusion*Chip*™, **without** the insert, into the hybridisation chamber (ref. FCHC-01)
- ❑ Denature ssDNA (**Vial T4**) (70µl per sample) by heating for **10 min at 100°C**, then immediately cool on ice for 5 min.
- ❑ Dispense **7 ml** of Hybridisation **Buffer B1** into the hybridisation chamber containing the *MLL* Fusion*Chip*™.
- ❑ Add **70 µl** of denatured ssDNA (100µg/ml final concentration) to the hybridisation chamber.
- ❑ Incubate the hybridisation chamber for **120 min at 65°C** in an oven, with orbital agitation.

8.1.2. Hybridisation

- ❑ **MIX** and boil the **two** probes (PCR II product) obtained from step 7.1.3, with 30µl ssDNA per sample, (i.e. for one sample, in the same tube: 50µl PCR II tube 1 + 50µl PCR II tube 2 + 70µl ssDNA) for **10 min at 100°C**.
- ❑ Immediately cool on ice for **5 min.**
- ❑ Centrifuge briefly (~10sec, 10,000rpm, to collect the liquid in the bottom of the tube).
- ❑ Remove the chip from the hybridisation chamber.
- ❑ Discard the pre-hybridisation buffer.
- ❑ Replace the *MLL* Fusion*Chip*™, **with** the insert, into the hybridisation chamber.

NB: Please note that the long edges of the insert's sides are not identical. One side is flat; the other has recessed edges. This distinction is easy to see by looking at the bottom end of the insert.
To place the MLL FusionChip into the chamber (hybridisation step 8.1.2 and detection step 8.2.3), hold the insert horizontally and lay the chip with the membrane facing up onto the flat side.
Carefully place the insert and chip into the container.
The hybridisation solution (or detection buffer, step 8.2.3) is added to the chamber by pipetting into the round opening at the top of the insert.

- ❑ Dispense **3 ml** of hybridisation **Buffer B1.**
- ❑ Add the **denatured probe** (with ssDNA) to the hybridisation chamber.
- ❑ Mix well.
- ❑ Incubate for **12-18 to 36 Hours at 65°C** in an oven, with orbital agitation.

8.1.3. Washing

- ❑ Check **Buffer B2** for SDS precipitation due to low storage temperature and, if necessary, dissolve by warming to 37°C.
- ❑ Remove the chip and the insert from the hybridisation chamber.
- ❑ Drain the hybridisation buffer.
- ❑ Replace the *MLL* Fusion*Chip*™, **without** the insert, into the hybridisation chamber.
- ❑ Rinse the chamber with washing **Buffer B2 (15 ml)** at room temperature.
- ❑ Renew the washing **Buffer B2 (15 ml)** and incubate the hybridisation chamber for **30 min at 65°C** in an agitating water bath or in an oven with orbital agitation.
- ❑ Prepare sufficient quantity of **1X Buffer B3** (90ml for one sample. 1/10 dilution in ddH_2O).
- ❑ Rinse **3 times for 5 min.** at room temperature with washing **Buffer B3 (15 ml)**, with agitation (agitating water bath or equivalent).

8.2. Colourimetric detection

8.2.1. Blocking and reaction with conjugate

- ❑ Prepare sufficient quantity of **1X Buffer B4** (30ml/sample. 1/5 dilution in ddH_2O).
- ❑ Drain the remaining B3 Buffer and dispense **15 ml** of blocking **Buffer B4** into the hybridisation chamber.
- ❑ **Incubate for 60 min at room temperature** with agitation.
- ❑ Renew (**15 ml**) the blocking **Buffer B4.**
- ❑ Add **3 µl** of **Vial T5** (streptavidine alkaline-phosphatase).
- ❑ Mix well.
- ❑ **Incubate the** hybridisation chamber **for 60 min at 37°C** with agitation (agitating water bath or equivalent).

8.2.2. Washing

- ❑ Drain the remaining blocking Buffer B4.
- ❑ Rinse with washing **Buffer B3 (15 ml)** at room temperature.
- ❑ Renew (**15 ml**) the washing **Buffer B3** and incubate for **15 min at room temperature with** agitation.
- ❑ Renew (**15 ml**) the washing **Buffer B3** and incubate for **15 min at 37°C with** agitation (agitating water bath or equivalent).

8.2.3. Detection

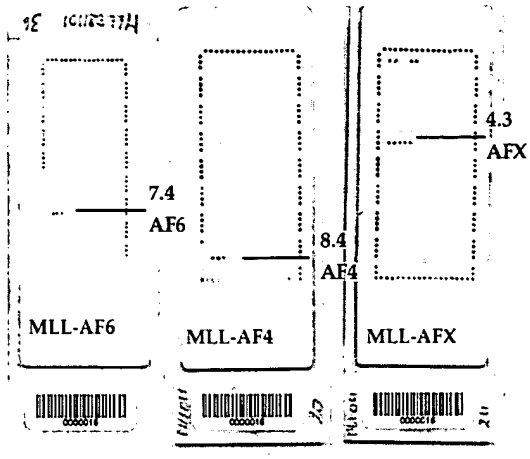
- ❑ Preheat buffer B5 to room temperature.
- ❑ Carefully drain the **Buffer B3** and <u>return the insert back</u> into the Hybridisation Chamber with the chip (see 8.1.2).
- ❑ Add **3 ml** of NBT/BCIP **(Buffer B5) and incubate at room temperature** in the dark.
- ❑ Carefully control the detection and stop the reaction after 15 minutes maximum.
- ❑ Incubate the slide for **10 min at room temperature** in a 10% acetic acid solution.
  The inverted L shape visualisation control should be visible at the end of the reaction process.

9. Test interpretation

Dark spots (figure 1 / page 5) visualise the hybridisation reactions between the sample and spotted oligonucleotides. An inverted L on the right of the chip confirms control of the detection reaction (when the chip is visualised with the label at the bottom). These dark spots define a grid, which identifies specific hybridisation reactions. Results are interpreted by comparing the processed chip with the template (figure 1) to identify each positive spot and the corresponding hybridised oligonucleotides. Below are practical examples, including non-interpretable test results.

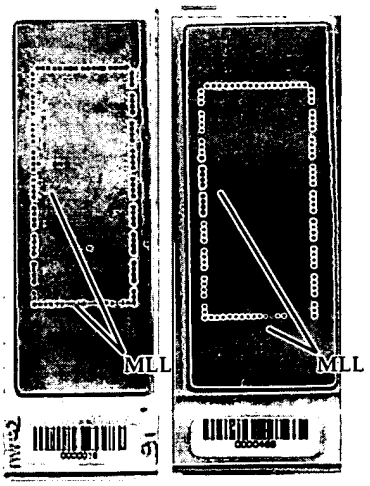
9.1. Positive test: *MLL*+ partner

When the L shape *MLL* pattern is complete or partial, and when at least one spot appears for one of the lines (corresponding to the spotted partner genes), the test is positive, i.e. the tested RNA carries an abnormal *MLL* transcript (see examples).



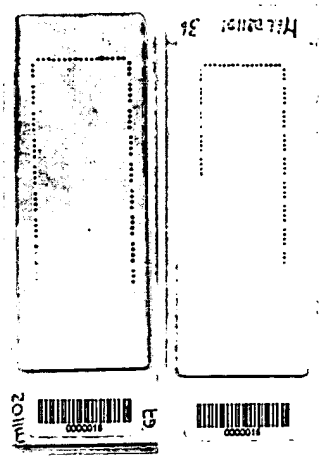
9.2. Negative test: normal *MLL* only

When the L shape *MLL* pattern is complete (at least to the first spots of the horizontal line), and when no signal appears for any of the spotted partner genes, the test is negative, i.e. the test RNA carries only normal *MLL* transcripts (see example).



9.3. Non interpretable test: partial *MLL*, no partner

When the L shape *MLL* pattern is not complete, and when no signal appears for any of the spotted partner genes, the test cannot be interpreted, even if the inverted L shape control correctly appears (see example).



10. Troubleshooting guide

Problem	Probable cause (s)	Suggested Corrective Action (s)
No control staining (Inverted L)	• Reagent omission (Vial T5)	• Review application of reagents
	• Insufficient incubation time (Buffer B5)	• Review application of reagents Repeat the visualisation step
	• Excessive dilution of the visualisation buffer (Buffer B5) in washing buffer B3	• Carefully drain the remaining buffer B3 prior adding Buffer B5
No or weak staining of Chip	• Reagents not used in proper order	• Review application of reagents
	• Poor quality sample RNA	• Assess RNA integrity, by denaturing agarose gel electrophoresis for example, before running array experiment. Degraded RNA may be visible as a smear • We recommend storing RNA at -70°C
	• Starting concentration of your experimental RNA is too low (< 1 µg)	• Quantify the RNA in a spectrophotometer by measuring O.D. at A_{260}
	• Pipetting errors, inverted (primers) or omitted reagents	• Check for missing reagents, follow strictly the pipetting scheme
	• Low biotin incorporation	• Check for missing reagents, repeat experiment as stated in product insert
	• Reagent (Buffer B5) incubation time too short	• Review application of reagents and perform the procedure as stated in the product insert
	• Reverse transcriptase and Taq Polymerase with poor activity • Enzyme past expiry date • Enzyme not stored at -20°C	• Repeat cDNA preparation and PCR with new enzyme • Store new enzyme at -20° • Perform a CQ on enzyme batch (cf. p12)
Excessive background	• Insufficient washing • Omission of a wash step • Incorrect wash temperature or length of wash shortened	• Review application of reagents and perform the procedure as stated in the product insert
	• Reagent (Buffer B5) incubation time too long	• Review application of reagents and perform the procedure as stated in the product insert
	• Inappropriate wash solution used	• Use only the wash buffers that are supplied with the kit
	• Length of blocking buffer (Buffer B4) pre incubation shortened • Incorrect temperature of blocking buffer (Buffer B4) incubation	• Review application of reagents and perform the procedure as stated in the product insert
	• Poor pre-hybridisation step • Pre-hybridisation step length was less than stated in product insert • Inadequate mixing of pre-hybridisation solution with the chip	• Review application of reagents and perform the procedure as stated in the product insert
	• Hybridisation solution was not pre-warmed to 65°C to dissolve the precipitate that forms at lower temperature	• Review application of reagents and perform the procedure as stated in the product insert
	• Inappropriate hybridisation solutions used	• Use only the hybridisation buffer (Buffer B2) that is supplied with the kit
	• Probes were added directly during the pre-hybridisation step	• Review application of reagents and perform the procedure as stated in the product insert
	• Reagent omission (SSDNA Vial T4)	• Review application of reagents and perform the procedure as stated in the product insert
High localised background	• The probe was added directly on the chip	• Dilute the probe in hybridisation buffer as described in the instructions for use
Excessively strong staining. Too many spots observed	• Insufficient washing • Omission of a wash step • Incorrect wash temperature or length of wash shortened	• Perform all wash steps as stated in the product insert • Review application of reagents and perform the procedure as stated in the product insert
	• Chip was allowed to dry out partially during the procedure • Chip was not completely submerged in buffer throughout the entire procedure	• Always keep chip submerged in wash solutions, except during buffer changes

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please contact IPSOGEN Technical Service for further assistance.

11. Performance Characteristics: Non-clinical studies

Non-clinical studies were conducted to establish the analytical performance of the *MLL* Fusion*Chip*™. These non-clinical laboratory studies were performed on cell lines total RNA from K562, U937, Karpas 45, ML2, and MV 4-11. Evaluated parameters include limit of detection and precision of the *MLL* Fusion*Chip*™.

11.1. Limits of Detection

The limit of detection for the *MLL* Fusion*Chip*™ was determined by testing dilutions of a positive RNA (MV 4-11 total RNA) within a negative RNA (U937 total RNA). Tested dilutions were 50%, 20% and 10% of positive RNA. The three dilutions were tested in parallel by a minimum of 3 different operators, and all of them gave a positive MLL-AF4 result on our *MLL* Fusion*Chip*™. These results are consistent with the percentage of blasts present at the time of diagnosis of acute leukaemia crisis (minimum 20% of blasts in a bone marrow sample).

MV 4-11 Total RNA / U937 total RNA	0%	10%	20%	50%	100%
100%	Neg				
90%		Pos			
80%			Pos		
50%				Pos	
0%					Pos

Neg: Normal MLL
Pos: MLL + Partner (AF4)

11.2. Precision studies

11.2.1. Repeatability

Intra-assay precision was tested by one global assessment of the same positive RNA (corresponding to total RNA extracted from MV4-11 cells) by individual operators. Samples were divided into 6 reverse transcriptions, and each resulting cDNA divided and amplified in parallel PCR I reactions. Each resulting PCR I product was subsequently divided and amplified in parallel PCR II labelling reactions. The 24 resulting probes were hybridized onto 24 individual *MLL* Fusion*Chips*™. The 24 hybridisations yielded 100% identical results, positive for the MLL-AF4 translocation.

11.2.2. Reproducibility

Reproducibility was evaluated with 3 different experimenters performing the *MLL* Fusion*Chip*™ test in 3 individual experiment plans from 3 positive (Karpas45 expressing a MLL-AFX-1 fusion transcript, ML2 expressing a MLL-AF6 fusion transcript and MV4-11 expressing a MLL-AF4 fusion transcript) and 2 negative (U937 and K562) cell lines. The table below shows the obtained results.

	Number of *MLL* Fusion*Chips*™	**Interpretable results**	**True results**	**Reproducibility**
Negative cell lines	16	12	12	75%
Positive cell lines	24	22	21	88%
All	**40**	**34**	**33**	**83%**

11.2.3. Specificity & Sensitivity

The previously described experiment allows the following specificity and sensitivity calculation:

	***MLL* + partner**	**normal *MLL* transcript**	
Positive tests	true positives = 54	false positives = 0	**PPV = 100 %**
Negative tests	false negatives = 1	false positives = 0	**NPV = 92 %**
	Sensitivity = 95 %	**Specificity = 100 %**	

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13.Miscellaneous

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IDOC-MK0025-MLLFCRUOENGVer01

*MLL*Fusion*Chip*™ is covered by patent application FR 0213487.
This process is covered by Ipsogen patent application WO99/23251 and its foreign counterparts.

[1] Expand is a trademark of a member of the Roche group
[2] RNAseOUT is a trademark of Invitrogen

Ipsogen
CancerProfiler

MLL PARTNERS

- CDK6
- MLL DUP
- EEN
- ABI1
- AF6q21
- hCDCRel-1
- AF1q
- ELL
- ENL
- GRAF
- AF10
- AF17
- AF9
- AFX-1
- FBP17
- GAS7
- GMPS
- MSF
- RARA
- AF15q14
- AF1p
- AF3p21
- GPHN
- LAF4
- LARG
- LPP
- AF6
- SEPTIN
- LCX
- AF5q31
- AF4
- CBP
- P300

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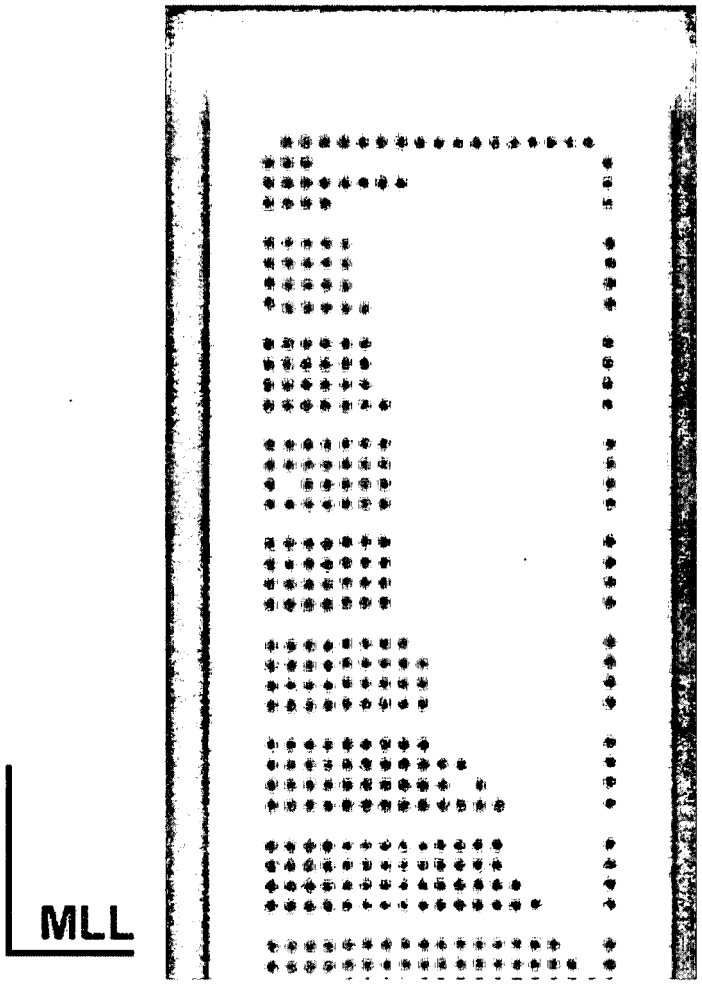
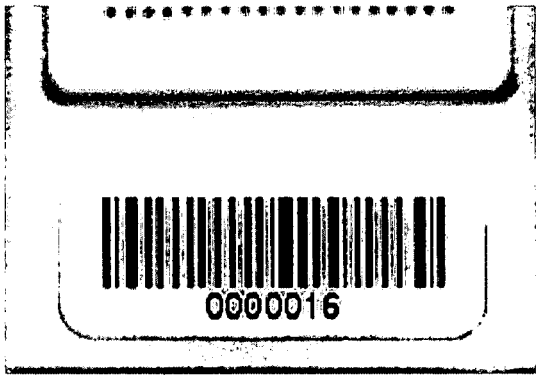
IPSOGEN *MLL* FUSIONCHIP™





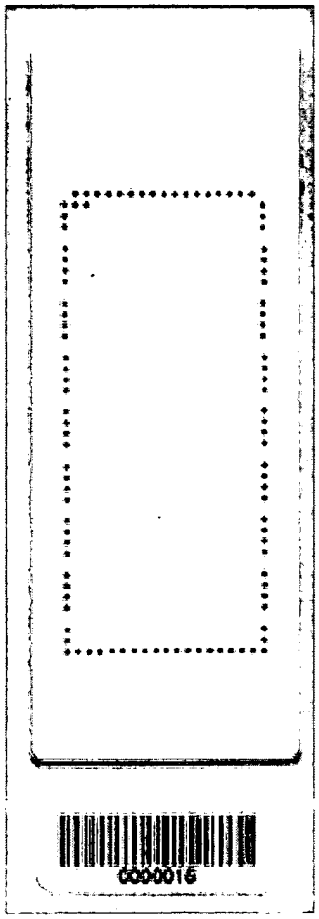
N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

The *MLL* Fusion*Chip* is a qualitative molecular diagnostic device that will confirm the presence of an MLL rearrangement, which gene is the fusion partner and the breakpoint position of the partner gene.

The protocol is based on the amplification and detection of fusion transcripts by means of a reverse transcription followed by an anchored PCR and hybridisation on the *MLL* Fusion*Chip*.

A colorimetric reaction visually shows the presence of an *MLL* translocation and allows the identification of the associated partner gene.

IPSOGEN *MLL* FUSION*CHIP*™

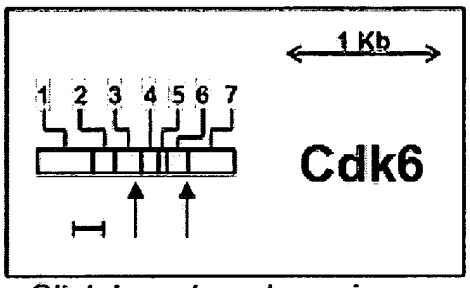


PARTNER: CDK6

POSITION: 7q21-q22

TRANSLOCATION:
t(11;7)(q23;q21)

OLIGO POSITIONING:



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IMPLICATION PATHO-MLL-CDK6:
Infant ALL.

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

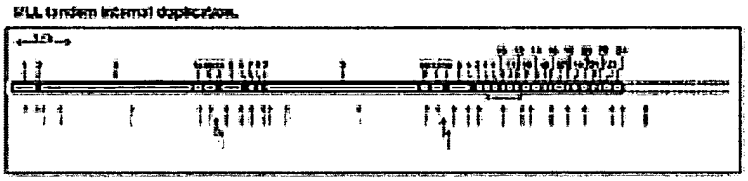
IPSOGEN *MLL* FUSION*CHIP*™

PARTNER: MLL DUPLICATION

POSITION:

TRANSLOCATION:

OLIGO POSITIONING:



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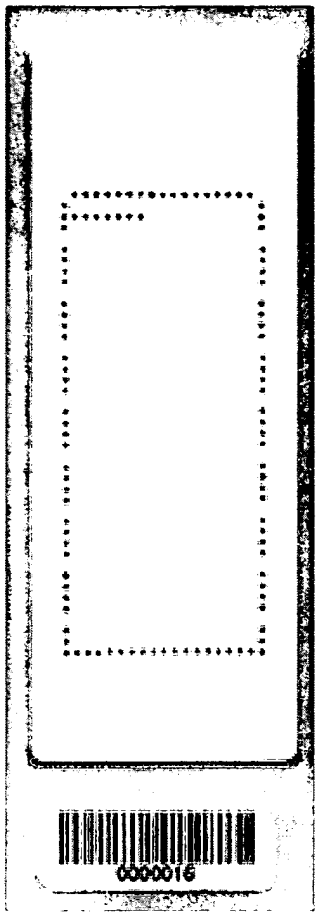
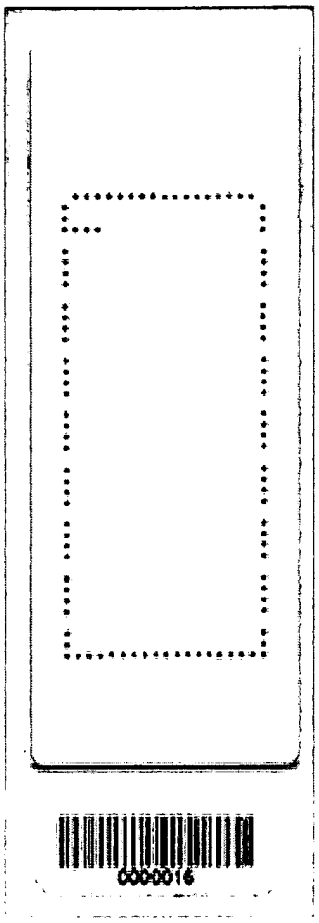


IMPLICATION PATHO-MLL-DUPLICATION:

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

IPSOGEN *MLL* FUSION*CHIP*™

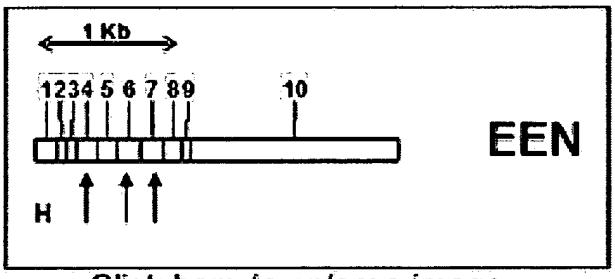


PARTNER: EEN

POSITION: 19p13

TRANSLOCATION:
t(11;19)(q23;p13)

OLIGO POSITIONING:



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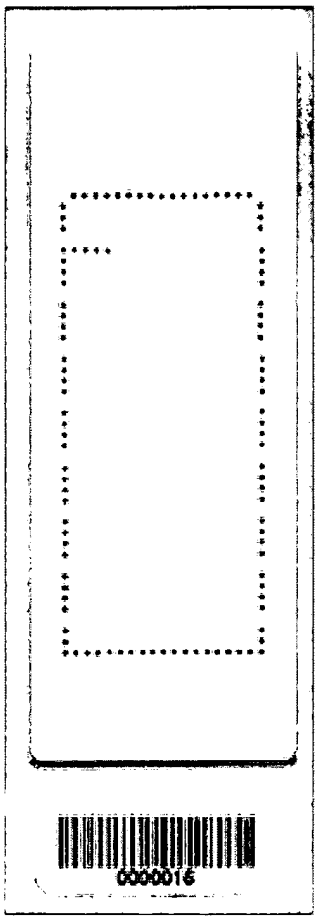
IMPLICATION PATHO-MLL-EEN:
Yet unknown (only one case).

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

IPSOGEN *MLL* FUSION*CHIP*™

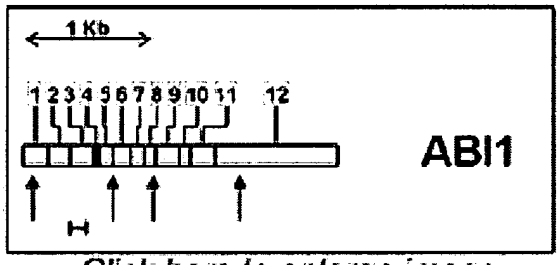


PARTNER: ABI1

POSITION: 10p12

TRANSLOCATION:
t(10;11)(p11.2;q23)

OLIGO POSITIONING:



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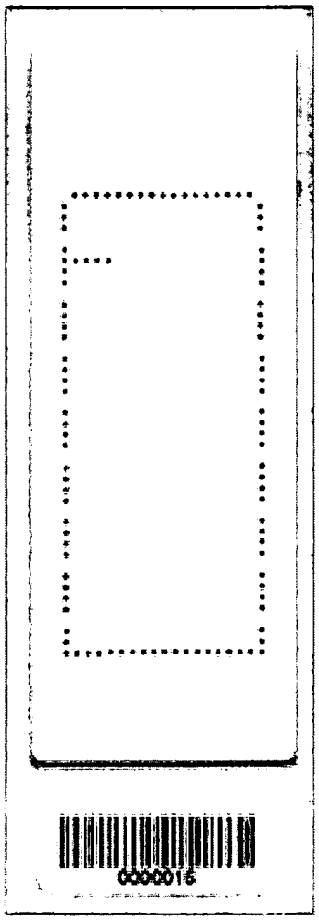
IMPLICATION PATHO-MLL-ABL1:
M4ANLL in an infant

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

IPSOGEN *MLL* FUSION*CHIP*™

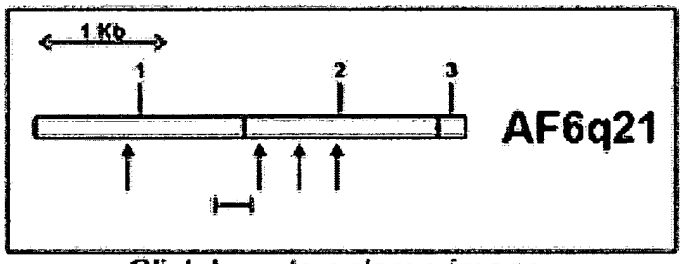


PARTNER: AF6q21

POSITION: 6q21

TRANSLOCATION:
t(6;11)(q21;q23)

OLIGO POSITIONING:



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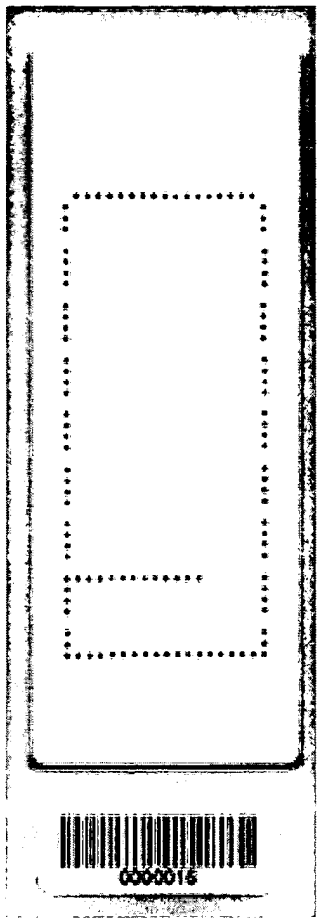
IMPLICATION PATHO-MLL-AF6Q21:
Treatment relative ANNL, M5 type.

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

IPSOGEN *MLL* FUSION*CHIP*™

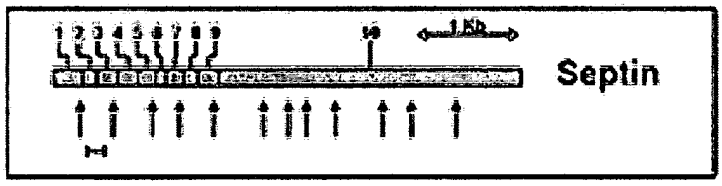


PARTNER: SEPTIN

POSITION: Xq22

TRANSLOCATION:
t(X;11)(q22;q23)

OLIGO POSITIONING:

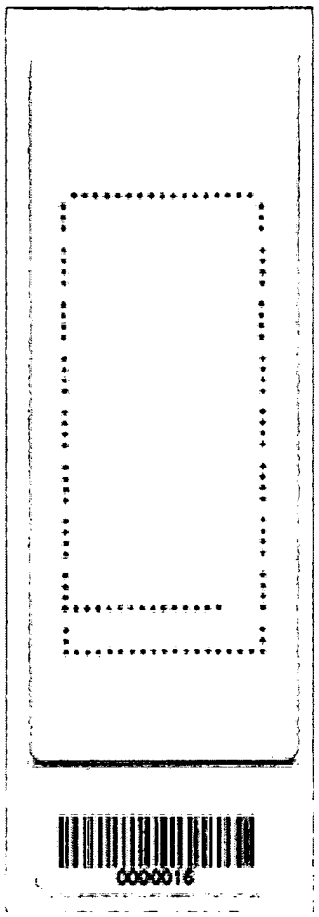


Click here to enlarge image

IMPLICATION PATHO-MLL-SEPTIN:
One case of infant AML.

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

IPSOGEN *MLL* FUSION*CHIP*™

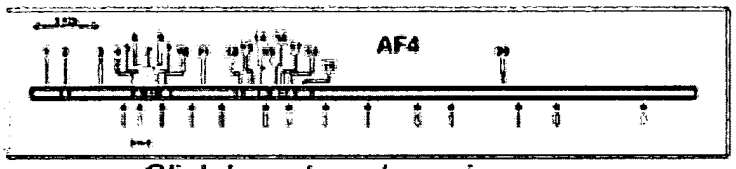


PARTNER: AF4

POSITION: 4q21

TRANSLOCATION:
t(4;11)(q21;q23) Variable Breakpoints.

OLIGO POSITIONING:



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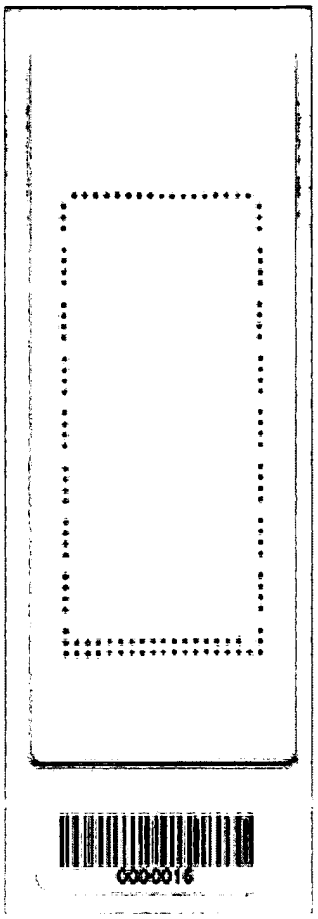
IMPLICATION PATHO-MLL-AF4:
Acute leukemia. Typically CD19+ B-ALL, biphenotypic AL, at times ANLL (M4/M5). Common in infant.
Treatment related leukemia.

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity

IPSOGEN *MLL* FUSION*CHIP*™

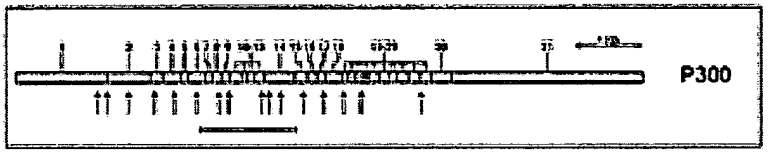


PARTNER: P300

POSITION: 22q13.2

TRANSLOCATION:
t(11;22)(q23;q13)

OLIGO POSITIONING:



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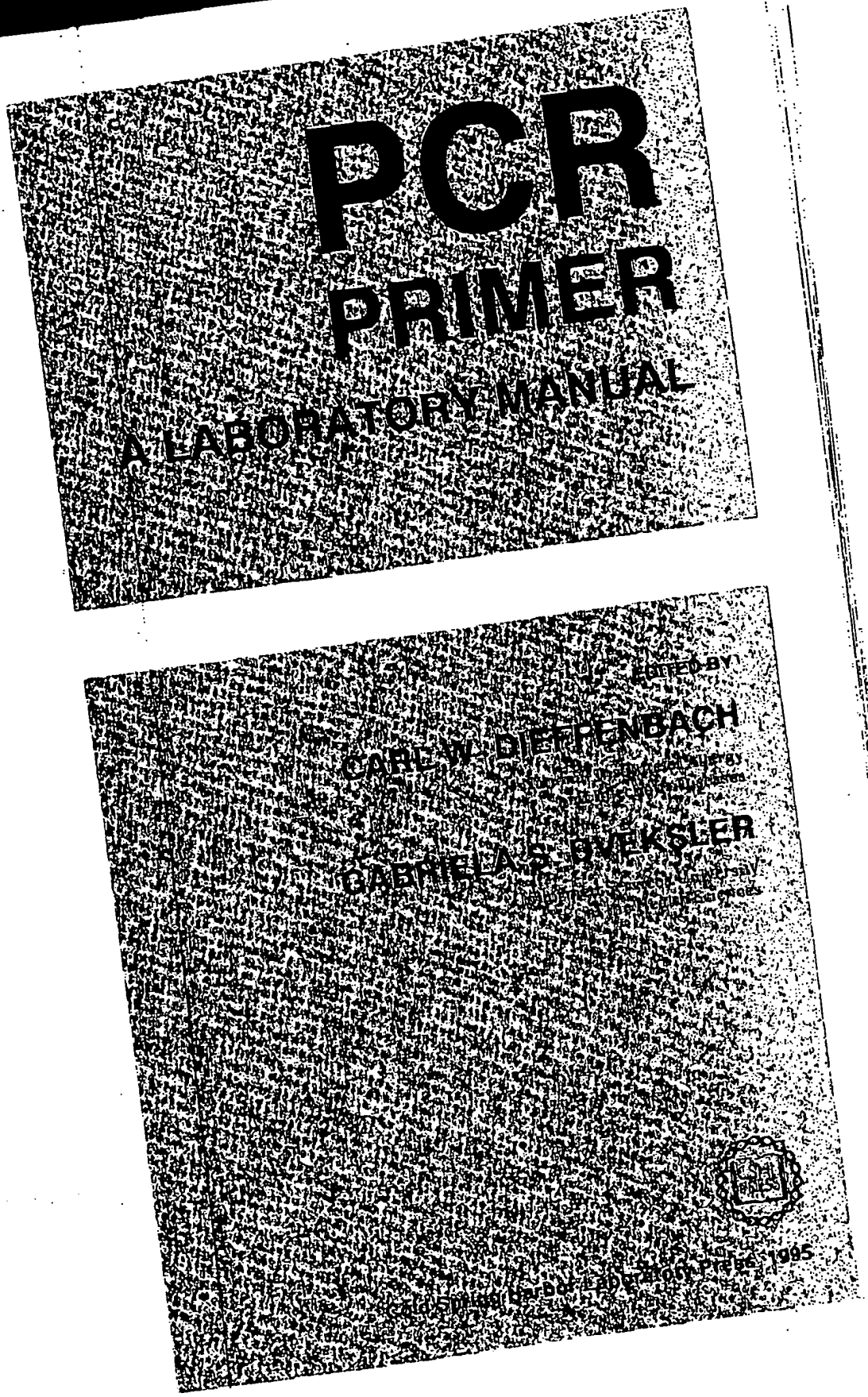
IMPLICATION PATHO-MLL-P300:
Very rare. Therapy related ANLL.

N.B. The actual biochip results will be seen in one color and are represented here in several colors purely for clarity


PCR
PRIMER
A LABORATORY MANUAL
EDITED BY
CARL W. DIEFFENBACH
GABRIELA S. DVEKSLER
Cold Spring Harbor Laboratory Press 1995

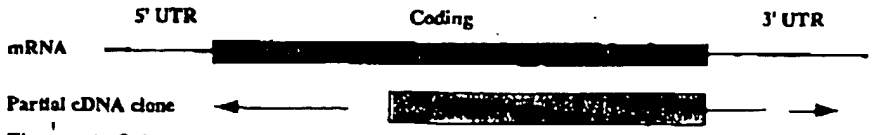


Figure 1 Schematic representation of the setting in which RACE is useful in cDNA cloning strategies. Depicted is an mRNA for which a cDNA representing only an internal portion of the transcript has been obtained. Such circumstances often arise; one such example is when closely related genes are cloned using PCR amplification with degenerate primers encoding sequences homologous to amino acids found in all known members of the gene family.

and observed. In addition, essentially unlimited numbers of independent clones can be generated using RACE, unlike library screens in which generally a single to a few cDNA clones are recovered. The availability of large numbers of clones provides confirmation of nucleotide sequence and allows the isolation of unusual transcripts that are alternately spliced or that begin at infrequently used promoters.

Classic RACE

PCR is used to amplify partial cDNAs representing the region between a single point in a mRNA transcript and its 3′ or 5′ end (Fig. 2). A short internal stretch of sequence must already be known from the mRNA of interest. From this sequence, gene-specific primers are chosen that are oriented in the direction of the missing sequence. Extension of the partial cDNAs from the unknown end of the message back to the known region is achieved using primers that anneal to the preexisting poly(A) tail (3′ end) or to an appended homopolymer tail (5′ end). Using RACE, enrichments on the order of 10^6- to 10^7-fold can be obtained. As a result, relatively pure cDNA "ends" are generated that can be easily cloned or rapidly characterized using conventional techniques (Frohman et al. 1988).

To generate "3′-end" partial cDNA clones, mRNA is reverse-transcribed using a "hybrid" primer (Q_T) that consists of 17 nucleotides of oligo(dT) followed by a unique 35-base oligonucleotide sequence (Q_I-Q_O; Fig. 2a, c), which in many reports is denoted as an "anchor" primer. Amplification is then performed using a primer containing part of this sequence (Q_O) that now binds to each cDNA at its 3′ end, and using a primer derived from the gene of interest (GSP1). A second set of amplification cycles is then carried out using "nested" primers (Q_I and GSP2) to quench the amplification of nonspecific products. To generate "5′-end" partial cDNA clones, reverse transcription (primer extension) is carried out using a gene-specific primer (GSP-RT; Fig. 2b) to generate first-strand products. Then, a poly(A) tail is appended

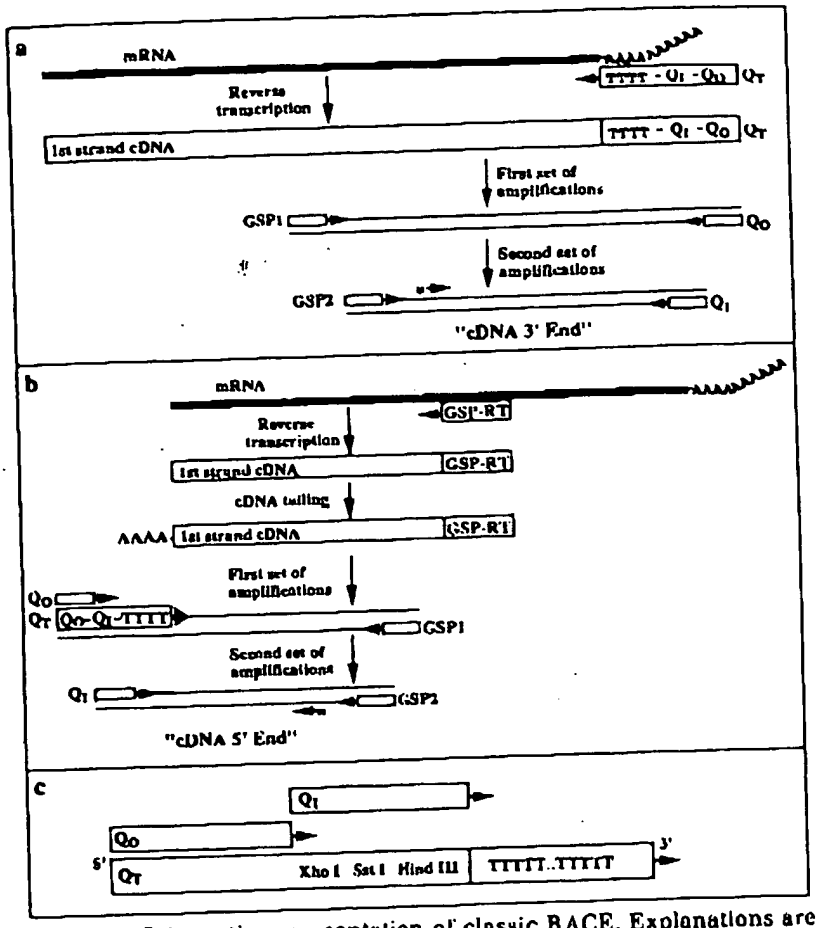


Figure 2 Schematic representation of classic RACE. Explanations are given in the text. At each step, the diagram is simplified to illustrate only how the new product formed during the previous step is utilized. (GSP1) Gene-specific primer 1; (GSP2) gene-specific primer 2; (GSP-RT) gene-specific primer used for reverse transcription; (*→) GSP-Hyb/Seq or gene-specific primer for use in hybridization and sequencing reactions. (*a*) Amplification of 3′-partial cDNA ends. (*b*) Amplification of 5′-partial cDNA ends. (*c*) Schematic representation of the primers used in classic RACE. The 52-nucleotide Q_T primer (5′-Q_O-Q_I-TTTT-3′) contains a 17-nucleotide oligo-(dT) sequence at the 3′ end followed by a 35-nucleotide sequence encoding *Hin*dIII, *Sst*I, and *Xho*I recognition sites. The Q_I and Q_O primers overlap by 1 nucleotide; the Q_I primer contains all three of the recognition sites. Primers: Q_T: 5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTT-TTTTTTTTTTTT-3′; Q_O: 5′- CCAGTGAGCAGAGTGACG-3′; Q_I: 5′-GAGGA-CTCGAGCTCAAGC-3′.

using terminal deoxynucleotidyltransferase (TdT) and dATP. Amplification is then achieved using (1) the hybrid primer Q_T to form the second strand of cDNA, (2) the Q_O primer, and (3) a gene-specific

primer upstream of the one used for reverse transcription. Finally, a second set of PCR cycles is carried out using nested primers (Q_t and GSP2) to increase specificity (Frohman and Martin 1989).

Classic RACE Variations

In general, as described above, the gene-specific primer is derived from a short stretch of sequence that is already known from the mRNA of interest. A frequent question is whether degenerate primers, i.e., primers directed against a predicted nucleotide sequence based on known amino acid sequence, can be used instead. Although such primers increase the quantity of spurious amplification, the approach can work, if other parameters are favorable (i.e., message abundance, GC composition, and cDNA end size; see Monstein et al. 1993).

At the unknown end of the cDNA, the 5′ end can be tailed with Cs instead of As and then amplified using a hybrid primer with a tail containing Gs (Loh et al. 1989) or a mixture of Gs and inosines (I) (Schuster et al. 1992). Although the G:I approach entails synthesizing a primer that can be used for 5′ RACE only (since a T-tailed primer must be used to anneal to the poly(A) tail of the 3′ end), there may be sufficient benefits from using a mixed G:I tail to justify the cost, since the G:I region should anneal at temperatures similar to those of other primers normally used in PCR. In contrast, it is believed that homopolymers of either Ts or Gs present problems during PCR, due to the very low and very high annealing temperatures, respectively, required for their optimal usage (Frohman et al. 1988; Schuster et al. 1992). On the other hand, the inosine residues function as degenerate nucleotides and lead to higher spurious amplification, so the magnitude of the benefit of using a mixed G:I primer is unknown.

To minimize the length of homopolymer tail actually amplified, a lock-docking primer was developed by Borson et al. (1992). In this approach, the final 2 nucleotides on the 3′ end of the primer are degenerate. For example, to amplify cDNAs linked to an A-tail, the lock-docking primer would look like:

$$5'\ \text{XXXXXXXXXX-AAAAAAAAA-}\begin{pmatrix}G\\T\\C\end{pmatrix}\begin{pmatrix}G\\A\\T\\C\end{pmatrix}\ 3'$$

where X represents (e.g.) one or more restriction sites at the 5′ end of the primer. The advantage of this approach is that it forces the primer to anneal to the junction of the natural or appended homopolymer tail and the cDNA sequence. The disadvantage is that it is necessary to synthesize four primers, because most synthesizers can only synthesize primers starting from an unambiguous 3′ end.

In another variation, the location of the anchor primer is changed from the end of the unknown region of sequence to random points within the unknown region (Fritz et al. 1991). This is accomplished using a primer containing an anchor region followed by six random nucleotides (5′-XXXXXXX-NNNNNN-3′) either for reverse transcription (3′ RACE) or for creation of the second strand of cDNA (5′ RACE). This approach is valuable when the 3′ or 5′ ends lie so far away from the region of known sequence that the entire unknown region cannot be amplified effectively. Using this approach, cDNA ends of defined sizes are not generated; instead, one obtains a library of randomly sized fragments, all of which initiate at the gene-specific primer. The largest fragments can be cloned and characterized, extending the length of the known sequence, and the process (or standard RACE) can be repeated until the real unknown end is identified. The development of "long" PCR may make this approach unnecessary.

New RACE

The most technically challenging step in classic 5′ RACE is to cajole reverse transcriptase to copy the mRNA of interest in its entirety into first-strand cDNA. Because prematurely terminated first-strand cDNAs are tailed by terminal transferase just as effectively as full-length cDNAs, cDNA populations composed largely of prematurely terminated first strands result primarily in the amplification and recovery of cDNA ends that are not full length either (Fig. 3a). This problem is encountered routinely for vertebrate genes, which are often quite GC-rich at their 5′ ends and thus frequently contain sequences that hinder reverse transcription. A number of laboratories have developed steps or protocols designed to approach the problem (Tessier et al. 1986; Mandl et al. 1991; Volloch et al. 1991; Brock et al. 1992; Bertrand et al. 1993; Fromont-Racine et al. 1993; Liu and Gorovsky 1993; Sallie 1993); the "new RACE" protocol described here is for the most part a composite adapted from the cited reports.

New RACE departs from classic RACE in that the "anchor" primer is attached to the 5′ end of the mRNA *before* the reverse transcription step; hence, the anchor sequence becomes incorporated into the first-strand cDNA if and only if the reverse transcription proceeds through the entire length of the mRNA of interest (and through the relatively short anchor sequence), as shown in Figure 3b.

Before beginning new RACE (Fig. 4a), the mRNA is subjected to a dephosphorylation step using calf intestinal phosphatase (CIP). This step actually does nothing to full-length mRNAs, which have methyl-G caps at their termini, but it does dephosphorylate degraded mRNAs, which are uncapped at their termini (Volloch et al. 1991). This makes

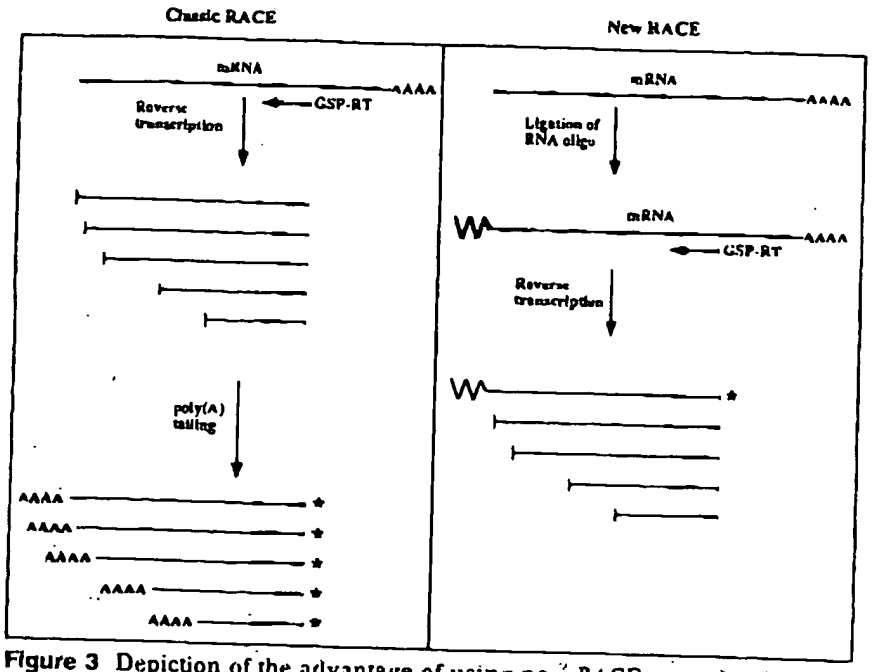


Figure 3 Depiction of the advantage of using new RACE over classic RACE. In classic RACE, premature termination in the reverse transcription step results in polyadenylation of less-than-full-length first-strand cDNAs, all of which can be amplified using PCR to generate less-than-full-length cDNA 5′ ends. * indicates cDNA ends created that will be amplified in the subsequent PCR. In new RACE, less-than-full-length cDNAs are also created, but are not terminated by the anchor sequence, and hence cannot be amplified in the subsequent PCR.

the degraded RNA biologically inert during the ensuing ligation step, because the phosphate group is required to drive the reaction. The full-length mRNAs are then decapped using tobacco acid pyrophosphatase (TAP), which leaves them with an active and phosphorylated 5′ terminus (Mandl et al. 1991; Fromont-Racine et al. 1993). Using T4 RNA ligase, this mRNA is then ligated to a short synthetic RNA oligonucleotide that has been generated by in vitro transcription of a linearized plasmid (Fig. 4b) (Tessier et al. 1986). The RNA oligonucleotide-mRNA hybrids are then reverse-transcribed using a gene-specific primer or random primers to create first-strand cDNA. Finally, the 5′ cDNA ends are amplified in two nested PCR procedures using additional gene-specific primers and primers derived from the sequence of the RNA oligonucleotides.

The new RACE approach can also be used to generate 3′ cDNA ends (Volloch et al. 1991; see also related protocols in Mandl et al. 1991 and Brock et al. 1992) and is useful in particular for non-polyadenylated RNAs. In brief, cytoplasmic RNA is dephosphorylated

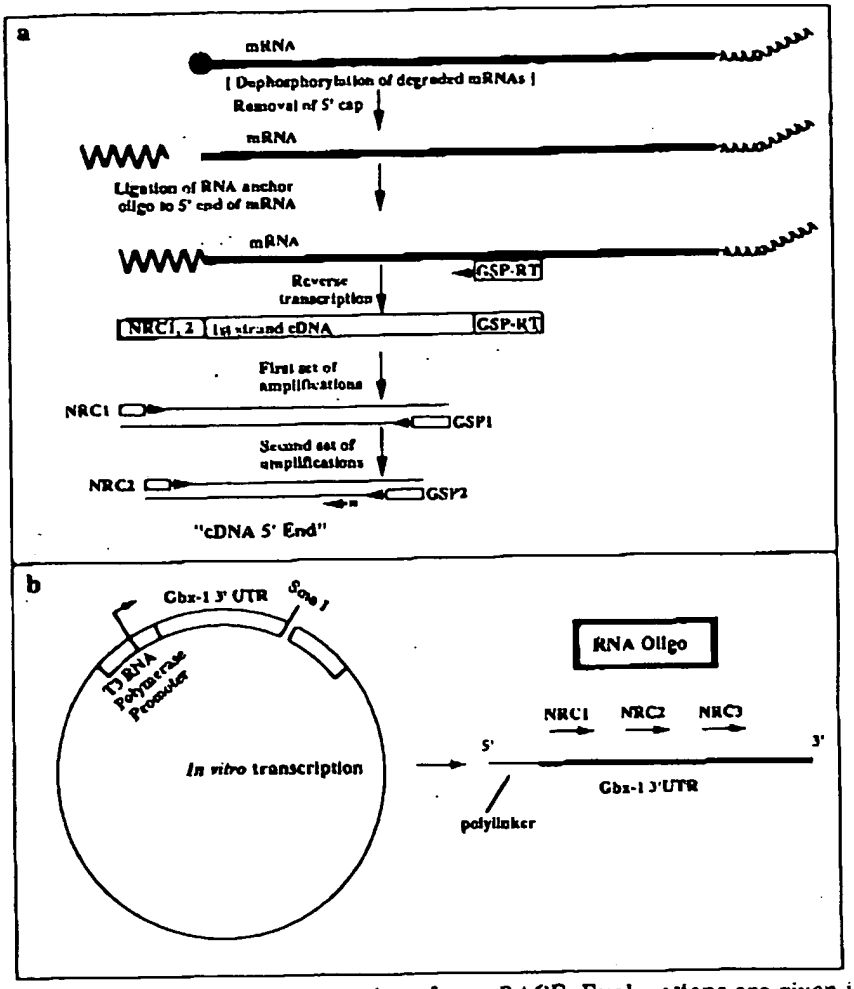


Figure 4 Schematic representation of new RACE. Explanations are given in the text. At each step, the diagram is simplified to illustrate only how the new product formed during the previous step is utilized. See legend to Fig. 2 for description of some primers. (*a*) Amplification of 5′-partial cDNA ends. (*b*) In vitro synthesis of the RNA oligonucleotide used for ligation in new RACE and schematic representation of the corresponding required primers. A 132-nucleotide RNA oligonucleotide is produced by in vitro transcription of the plasmid depicted using T3 RNA polymerase. Primers NRC-1, -2, and -3 are derived from the sequence of the oligonucleotide but do not encode restriction sites. To assist in the cloning of cDNA ends, the sequence ATCG is added to the 5′ end of NRC-2, as described in the cloning section of the text.

and ligated to a short synthetic RNA oligonucleotide as described above. Although ligation of the oligonucleotide to the 5′ end of the RNA was emphasized above, RNA oligonucleotides actually ligate to both ends of the cytoplasmic RNAs. For the reverse transcription step,

a primer derived from the RNA oligonucelotide sequence is used (e.g., the reverse complement of NRC-3, Fig. 4). Reverse transcription of the RNA oligonucleotides that happen to be ligated to the 3′ end of the cytoplasmic RNAs results in the creation of cDNAs that have the RNA oligonucleotide sequence appended to their 3′ end. Gene-specific primers oriented in the 5′→ 3′ direction and new RACE primers (e.g., the reverse complements of NRC-2 and NRC-1, Fig. 4) can be used in nested PCR to amplify the 3′ ends.

PART I: CLASSIC RACE

REAGENTS

The materials required for this procedure can be purchased, along with the appropriate 5x or 10x enzyme reaction buffers, from most major suppliers.

SuperScript II reverse transcriptase (BRL)
RNase H (BRL)
RNasin (Promega)
Taq DNA polymerase
TdT (BRL or Boehringer Mannheim)
10x buffer: 670 mM Tris-HCl, pH 9.0, 67 mM $MgCl_2$, 1700 μg/ml BSA, and 166 mM $(NH_4)_2SO_4$
Oligonucleotide primer sequences as in Figure 2 legend
dNTPs as 100 mM solutions (Pharmacia or Boehringer Mannheim)

Enzymes are used as directed by the suppliers, except for *Taq* DNA polymerase. Instead of using the recommended reaction mixture, use the 10x buffer listed above; reaction conditions are altered as further described below (Frohman et al. 1988; Frohman 1994). Primers can be used "crude" except for Q_T, which should be purified to ensure that it is uniformly full length.

PROTOCOLS

3′-End cDNA Amplification

STEP 1. REVERSE TRANSCRIPTION TO GENERATE cDNA TEMPLATES

1. Assemble the following reverse transcription components on ice: 4 μl of 5x reverse transcription buffer (5x buffer contains 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mm $MgCl_2$), 1 μl of dNTPs (stock concentration is 10 mM of each dNTP), 2 μl of 0.1 M DTT, 0.5 μl of Q_T primer (100 ng/μl), and 0.25 μl (10 units) of RNasin.

2. Heat 1 μg of poly(A)$^+$ RNA or 5 μg of total RNA in 13 μl of water for 3 minutes at 80°C, cool rapidly on ice, and spin for 5 seconds in a microfuge.